CONSTRUCTION OF sgRNA EXPRESSION SYSTEM TARGETING THE BRCA2 GENE USING CRISPR-Cas 9 TECHNOLOGY

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

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TO WHOM IT MAY CONCERN

This is to certify that **Mr. Arinjeet Sarkar**, a student of the Department of Biotechnology, Sister Nivedita University, has successfully completed his long-term training at **ATGC Diagnostics Pvt. Ltd., West Bengal, India,** in the domain of Molecular Biology, from **4th January 2025 to 16th May 2025.**

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Mr. Sarkar demonstrated a high level of enthusiasm, responsibility, and dedication throughout the training. His ability to grasp new techniques quickly and apply them effectively in the laboratory was commendable. He maintained professionalism and contributed positively to the lab environment, both individually and as a team member.

We appreciate his efforts and wish him continued success in his academic and professional pursuits.

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled **"Construction of sgRNA Expression System Targeting the BRCA2 Gene Using CRISPR-Cas 9 Technology"** in partial fulfilment of the requirements for the award of the degree of **Masters of Science** in **Biotechnology** is an authentic record of my own work carried out during the period from **4th January 2025** to **16th May 2025** under the supervision of **Dr. Susanta RoyChoudhury,** director at ATGC Diagnostics Pvt. Ltd.

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This is to certify that the project report titled "**Construction of sgRNA Expression System Targeting the BRCA2 Gene Using CRISPR-Cas 9 Technology** ", submitted by Mr. Arinjeet Sarkar (Registration No. 230020475958 Enrolment No. 2321210002047) to the Department of Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by him for the partial fulfilment of the requirements for the Master of Science degree.

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ABBREVIATIONS

ACRONYM	DESCRIPTION
µg	Microgram
µl	Microlitre
µM	Micro molar
bot	Bottom
bp	Base pair
BRCA2E18	BRCA2 exon number 18
DNA	Deoxyribonucleic acid
DH5α	Douglas Hanahan's strain alpha
dNTP	Deoxyribonucleotide Triphosphate
E. Coli	Escherichia coli
EcoRI	Escherichia coli R strain Y13
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium bromide
HDR	homology-directed repair
L	Litre
LA	Luria-Bertani agar
LAF	Laminar Air Flow
LB	Luria-Bertani broth
mg	Milligram
Min	Minutes
ml	Millilitre
mM	Milli molar
NHEJ	Non-homologous End Joining
nt	Nucleotide
OD	Optical density
PAM	Protospacer Adjacent Motif
PCR	Polymerase chain reaction
psi	Per square inch
RNA	Ribo nucleotide
rpm	Revolution per minute
RT	Room temperature
sec	Seconds
Sg	Single guide
SLS	SLS Research Private Limited
TAE	Tris acetate EDTA
UV	Ultraviolet

ABSTRACT

The BRCA2 gene plays a critical role in maintaining genomic stability through homologous recombination-mediated DNA repair. Mutations in the BRCA2 gene are strongly associated with the increased susceptibility to breast, ovarian, and other cancers. Recent advancements in gene-editing technologies, particularly the CRISPR-Cas9 system, offer a promising approach to precisely target and modify disease-related genes. Central to the specificity of CRISPR-Cas9 is the single-guide RNA (sgRNA), which directs Cas9 to the intended genomic locus. This study aimed to construct an efficient sgRNA expression system specifically targeting the BRCA2 gene, to establish a platform for future therapeutic applications or functional studies.

The BRCA2 gene sequence was retrieved from NCBI and analysed to identify suitable protospacer adjacent motif (PAM) sites. Multiple candidate sgRNA sequences were designed using bioinformatics tools such as CHOPCHOP and assessed for off-target potential and on-target efficiency. The most suitable sgRNA was synthesized and cloned into a CRISPR-Cas9 expression plasmid vector, i.e. pSpCas9(BB)-2A-Puro V2.0 (PX459). The recombinant construct was then transformed into DH5α *Escherichia coli*, and positive colonies were screened using colony PCR using insert-specific and End point PCR using vector-specific primers. The presence and integrity of the insert was further validated through Sanger sequencing.

The results confirmed successful construction of the sgRNA expression system with high target specificity toward BRCA2. This study demonstrates a systematic approach to designing and constructing a gene-specific CRISPR-Cas9 tool, laying the groundwork for downstream applications.

KEYWORDS – BRCA2 gene, homologous recombination-mediated DNA repair, CRISPR-Cas9 system, single-guide RNA (sgRNA), Protospacer Adjacent Motif (PAM), Colony PCR, Sanger sequencing.

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1. INTRODUCTION

INTRODUCTION

Cancer remains a major global health concern, accounting for millions of deaths annually. Among various genetic contributors to cancer development, the BRCA2 (Breast Cancer Type 2 Susceptibility Protein) gene plays a pivotal role in DNA repair and tumour suppression. BRCA2 is located on chromosome 13q13.1 and encodes a protein that maintains genomic integrity through its involvement in homologous recombination (HR), a key mechanism in the repair of double-stranded DNA breaks (DSB). Loss-of-function mutations in BRCA2 compromise HR, pushing cells toward error-prone repair mechanisms such as non-homologous end joining (NHEJ), thereby increasing the likelihood of chromosomal abnormalities and oncogenic transformation. BRCA2 germline mutations are linked to a higher risk of pancreatic, ovarian, prostate, and breast cancers. The lifetime risk of breast cancer may be 69% higher for women with mutant BRCA2 variants. Consequently, *BRCA2* has become an important target for both diagnostic and therapeutic research.

The advent of genome editing technologies has revolutionized the ability to investigate gene function and develop gene-based treatments. Among these, the CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR associated protein 9) system has emerged as a powerful, programmable gene-editing platform. Derived from the adaptive immune system of *Streptococcus pyogenes*, CRISPR-Cas9 utilizes a single guide RNA (sgRNA) that allows precise targeting and modification of specific DNA sequences in eukaryotic cells. The single-guide RNA (sgRNA), which is at the very core of this system, uses complementary base pairing to guide the Cas9 endonuclease to the target DNA sequence. It comprises of a 20-nucleotide spacer sequence that is complementary to the target DNA and has a scaffold region that binds to Cas9. This sgRNA–Cas9 complex induces double-stranded breaks at the desired site, enabling gene disruption, repair, or replacement.

For CRISPR-mediated gene editing to be successful, an efficient sgRNA must be designed. The specificity and efficiency of the sgRNA directly determine the accuracy of the gene editing process and minimize off-target effects. A properly constructed sgRNA expression system allows for stable and controlled expression of the guide RNA in host cells, thus facilitating targeted genome modifications.

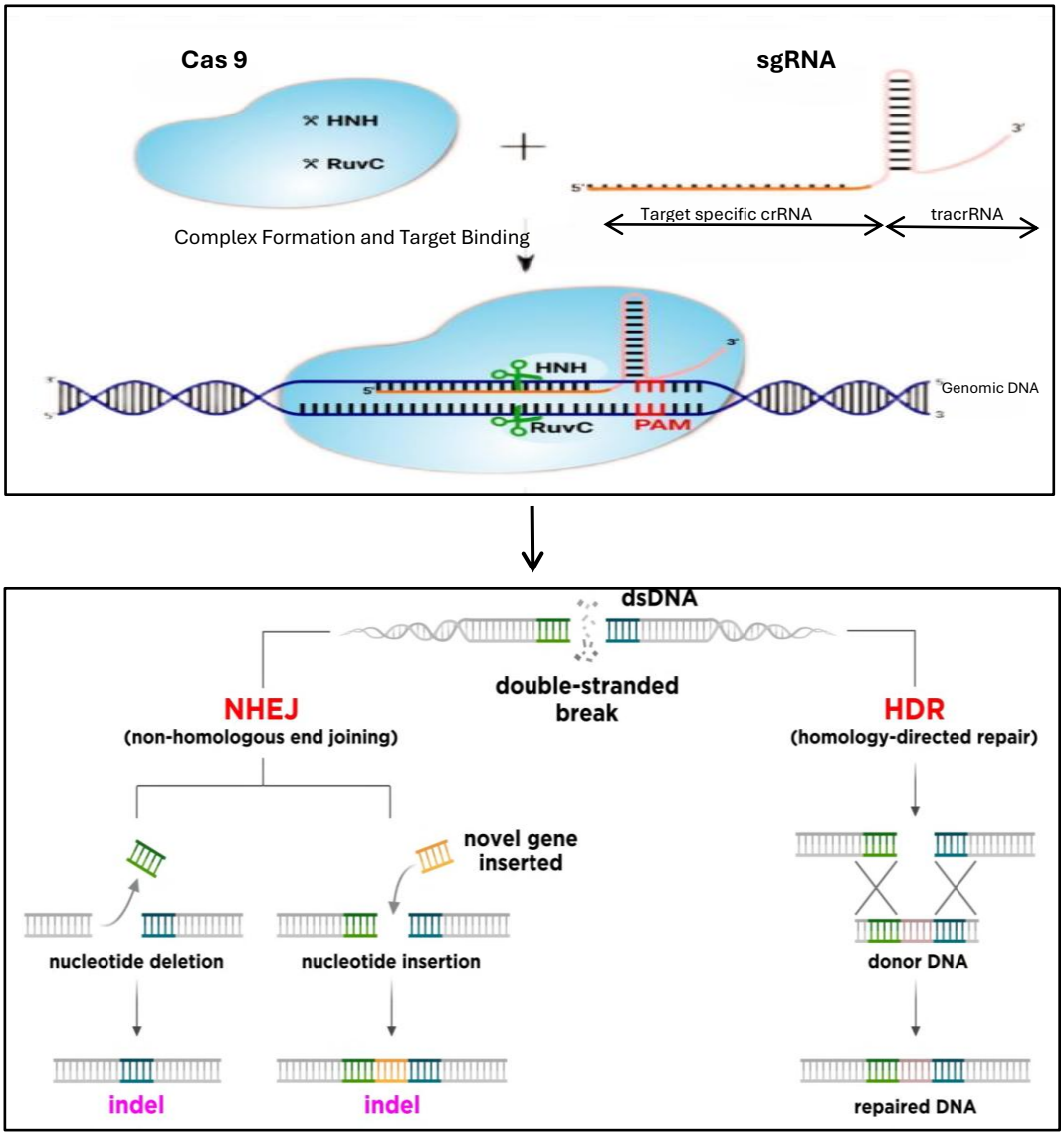


Fig 1.1: CRISPR-Cas 9 Mechanism

Cas9 Protein and Guide RNA: The image shows the Cas9 protein (blue shape) with two important nuclease domains HNH and RuvC. Alongside is the guide RNA (orange and black), which directs the Cas9 to a specific DNA sequence. The guide RNA binds to a complementary DNA sequence next to a short DNA motif called PAM (Protospacer Adjacent Motif, highlighted in red). Cas9, guided by the RNA, introduces a double-stranded break (DSB) at the target site. The HNH domain cuts one DNA strand, and the RuvC domain cuts the other. After Cas9 creates a DSB, the cell repairs the break using one of two main pathways i.e. Non-Homologous End Joining (NHEJ), the cell directly joins the broken DNA ends without using a template. Thus, this method is error-prone and often results in small insertions or deletions (indels), which can disrupt the target gene. Sometimes, a novel gene can be inserted at the break site. The repaired DNA may have mutations (indels) that can knock out gene function.

The repair mechanism is Homology-Directed Repair (HDR), Here the cell uses a homologous DNA template (donor DNA) to accurately repair the break. Which allows precise editing such as correcting a mutation or inserting a new gene sequence. The DNA is repaired accurately, incorporating the desired changes.

This dissertation focuses on the construction of a sgRNA expression system that targets a specific region in exon 18 of the BRCA2 gene, with a hypothesis of enabling precise gene manipulation for research or therapeutic purposes. The rationale behind this study lies in the potential application of gene-editing systems for knocking out dysfunctional alleles in cancer-prone individuals. Additionally, the system developed here may serve as a foundational tool for further studies into the functional domains of BRCA2 and its interaction with other proteins involved in DNA repair pathways. The goal is to design a high-efficiency, low off-target sgRNA sequence, and incorporate it into a CRISPR-Cas9 expression plasmid i.e. pSpCas9(BB)-2A-Puro (PX459) which is cloned with the help of a competent cell DH5α *Escherichia coli,* suitable for future gene editing applications. The approach involves bioinformatic analysis to identify potential PAM (Protospacer Adjacent Motif) sites within the *BRCA2* coding sequence, selection and validation of optimal sgRNA candidates, and molecular cloning to generate the final expression construct. The project encompasses several key steps: bioinformatic analysis of the BRCA2 gene to identify suitable target sites with adjacent PAM sequences retrieved from NCBI, design and selection of high-specificity sgRNA candidates using established algorithms like CHOPCHOP, cloning of the selected sgRNA sequence into a CRISPR-Cas9 expression vector using SnapGene, Designing of primers using bioinformatic tools like Addgene, NCBI primer BLAST and SnapGene, validation of the construct through molecular techniques including colony PCR, End point PCR and Sanger sequencing.

In conclusion, the targeted manipulation of BRCA2 using CRISPR-Cas9 represents a significant step toward personalized genetic medicine. This research provides a blueprint for constructing customized sgRNA systems for any gene of interest. As the field of genome engineering continues to evolve, the ability to design and validate effective sgRNA constructs will remain a cornerstone of future therapeutic developments.

2. REVIEW OF LITERATURE

REVIEW OF LITERATURE

The BRCA2 gene plays a critical role in maintaining genomic stability by facilitating homologous recombination repair of DNA double-strand breaks. Mutations in BRCA2, particularly in functional domains, are associated with increased susceptibility to breast and ovarian cancers. CRISPR-Cas9 technology has revolutionized genome editing, enabling precise targeting and modification of specific genomic loci. The construction of single guide RNA (sgRNA) expression systems targeting BRCA2 exon 18 is essential for functional studies and therapeutic applications. This review discusses the methodologies involved in designing and constructing of a sgRNA expression systems, focusing on transformation, digestion of plasmid, ligation of insert, plating techniques, colony PCR, primer designing, vector-specific endpoint PCR, and Sanger sequencing.

2.1. BRCA2 Gene and Exon 18: Functional Importance

The reference sequence for BRCA2 was retrieved from the National Center for Biotechnology Information (NCBI) database **(https://www.ncbi.nlm.nih.gov/gene/675)**. BRCA2 encodes a large protein involved in DNA repair through homologous recombination. Exon 18 is part of the C-terminal DNA-binding domain, critical for BRCA2's interaction with RAD51 and DNA repair function. Multiple studies have focused on exon 18 due to its involvement in pathogenic variants affecting protein function and cancer susceptibility **(Findlay et al., 2023; Findlay et al., 2025)**. Saturation genome editing (SGE) approaches have targeted exon 18 to systematically evaluate the functional impact of all possible single-nucleotide variants (SNVs), highlighting the importance of precise editing tools to dissect variant effects.

2.2. CRISPR-Cas9 Technology for Targeting BRCA2

CRISPR-Cas9 is a programmable RNA-guided DNA endonuclease system that introduces double-strand breaks at specific genomic loci. The system relies on an sgRNA that directs Cas9 to the target DNA sequence adjacent to a protospacer adjacent motif (PAM). The double-strand breaks induced by Cas9 are repaired by cellular pathways such as non-homologous end joining (NHEJ) or homology-directed repair (HDR), enabling gene disruption or precise sequence replacement **(Ran et al., 2013; Hsu et al., 2014)**.

For BRCA2, CRISPR-Cas9 has been employed to generate knockouts and to introduce specific mutations for functional studies. For example, CRISPR-mediated knockout of Brca2 exon 3 and exon 19 in mouse and human cell lines has been reported to model BRCA2 deficiency and study its role in tumorigenesis **(Jiang et al., 2016; Jonkers et al., 2019)**. These studies demonstrate the feasibility of targeting BRCA2 exons with sgRNAs designed using online tools such as CHOPCHOP **(https://chopchop.cbu.uib.no/)** and CRISPR Design.

2.3. Design of sgRNA Targeting BRCA2 Exon 18

The design of sgRNAs targeting BRCA2 exon 18 must consider several parameters to maximize editing efficiency and minimize off-target effects:

- **Target Sequence Specificity:** sgRNAs are selected to target unique sequences within exon 18, typically 20 nucleotides upstream of a PAM sequence (NGG for SpCas9).
- **On-Target Efficiency:** Computational tools predict sgRNA efficiency based on sequence features, secondary structure, and chromatin accessibility.
- **Off-Target Minimization:** sgRNAs with minimal sequence homology to other genomic regions are preferred to reduce unintended edits.
- **Position Relative to Functional Domains:** Targeting regions critical for BRCA2 function, such as DNA-binding motifs encoded by exon 18, ensures functional impact of edits.

Software tools like CHOPCHOP **(https://chopchop.cbu.uib.no/)** were used to generate multiple candidate sgRNAs for BRCA2 exons, including exon 18 (Jiang et al., 2016; Findlay et al., 2023)[3]. Optimal sgRNAs are then cloned into expression vectors such as PX459, which co-express Cas9 and the sgRNA under U6 promoters.

2.4. Vector Preparation: Digestion and Ligation

The cloning of sgRNA sequences into CRISPR-Cas9 vectors such as pSpCas9(BB)-2A-Puro (PX459) V 2.0.**(https://www.addgene.org/62988/)**, involves restriction digestion and ligation steps**(https://media.addgene.org/cms/filer_public/6d/d8/6dd83407-3b07-47db-8adb-4fada30bde8a/zhang-lab-general-cloning-protocol-target-sequencing_1.pdf)**. The vector is linearized using type IIS restriction enzymes like BbsI, which create compatible overhangs for sgRNA oligonucleotide insertion. Digestion is followed by purification to remove residual enzymes and small fragments.

2.5. Transformation and Replica Plating

The ligation mixture is transformed into chemically competent Escherichia coli (e.g., DH5α) via heat shock. Post-transformation, cells are plated on selective media containing antibiotics such as ampicillin to isolate transformants harboring the recombinant plasmid.

Replica plating is employed to transfer colonies from master plates to secondary selective plates for screening purposes without disturbing the original colonies. This technique facilitates high-throughput screening and identification of positive clones.

2.6. Primer Designing

Primer designing is integral for checking the integrity and sequence of the recombinant plasmid also to screen transformed colonies and confirm the presence of the sgRNA insert **(Jiang et al., 2016; Findlay et al., 2023)**.

2.7. Colony PCR and Vector-Specific Endpoint PCR

Colony PCR serves as a rapid screening method to verify the presence of the sgRNA insert in bacterial colonies. Using primers flanking the sgRNA insertion site, PCR amplification yields products of expected sizes only in colonies containing the recombinant plasmid **(Ran et al., 2013)**. Vector-specific endpoint PCR further confirms the integrity and orientation of the insert.

2.8. Sanger Sequencing for Validation

Sanger sequencing remains the gold standard for validating sgRNA constructs. Purified plasmids from positive colonies are sequenced using primers annealing to vector regions adjacent to the sgRNA insert. High-quality chromatograms confirm the correct sgRNA sequence, absence of mutations, and proper ligation **(Ran et al., 2013)**. This step is crucial to ensure that the sgRNA expression system will function as intended in mammalian cells, as sequence errors can lead to off-target effects or loss of editing efficiency

The construction of an sgRNA expression system targeting BRCA2 exon 18 using CRISPR-Cas9 technology involves a series of interconnected molecular biology techniques. Starting from bioinformatics-guided sgRNA and primer design, through vector digestion, ligation, bacterial transformation, and colony screening, to final validation by Sanger sequencing, each step is critical for ensuring the accuracy and efficiency of genome editing

3. MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 MATERIALS

Routine aseptic conditions were followed while performing all the experiments unless specified otherwise. All the media, solutions, reagents, disposable wares (tips, plates, tubes, spreaders, loops) were sterilised by autoclaving at 121ºC / 15 psi for 15 min.

3.1.1. Plasmid used (Provided by Ms. Anupa Chattopadhyay, Saroj Gupta Cancer Research Centre)

PLASMID	CONCENTRATION	REFERENCE
pSpCas9(BB)-2A-Puro (PX459) V2.0 [PX459]	100 ng/µl	https://www.addgene.org/browse/sequence/456946/

Table 3.1.1: List of Plasmids used.

3.1.2. *Escherichia coli* strain used

STRAIN	GENOTYPE
DH5α [SLS]	F- 80dlacZ M15 (lacZYA-argF) U169 recA1 endA1 hsdR17(rk- mk+) phoA supE44 -thi-1 gyrA96 relA1

Table 3.1.2: List of Strains used.

3.1.3. Antibiotic used

COMPONENTS	STOCK CONCENTRATION	USED CONCENTRATION
Ampicillin	1g	100 mg/ml

Table 3.1.3: List of antibiotics used.

3.1.4.: Luria-Bertani medium (LB) – 100 ml

COMPONENTS	AMOUNT
Luria Bertani [SRL]	2.5 g

Table 3.1.4: Components for preparing LB media.

To bring the volume up to 100 ml distilled water was added.

3.1.5. Luria-Bertani Agar (LA) – 100 ml

COMPONENTS	AMOUNT
Luria Bertani [SRL]	2.5 g
Agar agar [SRL]	1.5 g

Table 3.1.5: Components for preparing Luria-Bertani Agar (LA).

To bring the volume up to 100 ml distilled water was added.

3.1.6. 10X TAE Buffer

COMPONENTS	AMOUNT
Tris's base [SRL]	4.84 g
Glacial acetic acid [SRL]	1.14 ml
EDTA 0.5M (pH 8.0) [SRL]	1 ml

Table 3.1.6: Components for preparing 10X TAE buffer.

To bring the volume up to 100 ml distilled water was added.

3.2 METHODS

3.2.1. SINGLE GUIDE RNA (sgRNA) DESIGNING TARGETING BRCA2 GENE.

Single guide RNA (sgRNA) design is a critical step in CRISPR-Cas9 genome editing, as it determines the specificity and efficiency of the system. A scaffold sequence that attaches to the Cas9 protein and a 20-nucleotide spacer sequence that directs Cas9 to the target DNA site make up a synthetic RNA molecule known as a sgRNA. Effective sgRNA design involves selecting a target sequence adjacent to a protospacer adjacent motif (PAM), typically "NGG" for SpCas9, and minimizing off-target effects by ensuring sequence uniqueness in the genome. Bioinformatics tools such as CHOPCHOP are commonly used to design sgRNAs with high on-target activity and low off-target potential.

Requirements:

- Internet access
- Target DNA sequence in BRCA 2 gene (in FASTA format)
- sgRNA designing tool (CHOPCHOP)

Procedure:

1. Retrieval of the Target Gene Sequence:

a. Locating BRCA2 on NCBI:

- A Google search was conducted to access the NCBI website.
- In the NCBI search bar, the term “BRCA2” was entered.

b. Choosing the Appropriate Transcript:

- From the list of results, the BRCA2 transcript identified by RefSeq ID NM_000059.4 was selected.
- The corresponding GenBank entry was reviewed to obtain comprehensive sequence data.

c. Extracting the Exon 18 Sequence:

- The sequence for Exon 18 was located within the GenBank entry and its FASTA format was copied for downstream analysis.

2. Designing sgRNA Using CHOPCHOP:

a. Accessing CHOPCHOP:

- Google was used to navigate to the CHOPCHOP web tool.

b. Input of Target Sequence:

- The FASTA sequence for BRCA2 Exon 18 was pasted into the “Target” field on the CHOPCHOP interface.

c. Configuration of Parameters:

- The species was set to Homo sapiens.
- The application was specified as knock-out.

d. Identification of Target Sites:

- The "Find Target Sites!" button was clicked to initiate the sgRNA design process.

3. Evaluation and Selection of sgRNA Candidate

a. Result Interpretation:

- CHOPCHOP generated a list of potential sgRNA target sites within the input sequence, each ranked based on predicted specificity and efficiency.

- Information displayed for each sgRNA included:
    - Target sequence
    - Genomic coordinates
    - GC content
    - Self-complementarity
    - Predicted activity
    - Off-target analysis

b. Choosing the Optimal sgRNA:

- An sgRNA was selected based on:
    - High predicted cleavage efficiency
    - Minimal off-target effects
    - Appropriate GC content (ideally 40–60%)
    - Low self-complementarity to reduce the likelihood of secondary structure formation

4. Documentation and Synthesis of sgRNA

The highest-ranked sgRNA was chosen and converted into two oligonucleotides designed for cloning into the CRISPR-Cas9 vector pSpCas9(BB)-2A-Puro V2.0 (PX459). Overhangs compatible with BbsI restriction sites were added to enable directional cloning. The oligos were named as follows:

- BRCA2E18 Top Oligomer: 5'-**CACCG**CTTCATGGAGCAGAACTGGT–3' [25-nt sgRNA sequence]
- BRCA2E18 Bottom Oligomer: 5'-AAACACCAGTTCTGCTCCATG**AAGC**–3' [25-nt sgRNA sequence]

These oligonucleotides were ordered from **syngynx** using the names listed above.

3.2.2. PREPARATION OF COMPETENT CELLS (DH5α)

Competent cells are cells which are modified in such a manner that they can easily take up foreign DNA. There are several different methods to make cells competent like electroporation and treatment with calcium ions. Cells are made competent through calcium chloride method by suspending them in a solution containing concentration of calcium and thus creating pores in bacterial cells to take up the foreign DNA. Competency is better suited for cells when they are in their log phase.

Requirements:

- E. coli strain [DH5α]
- Luria-Bertani (LB) medium (Table 3.1.4)
- Centrifuge [Hermle]
- Incubator [sb equipment]
- Solutions for competent cell preparation-

COMPONENTS	AMOUNT
Transformation Buffer [SRL]	30 ml stock

Table 3.2.2: Components needed for preparation of competent DH5α cell.

Procedure:

1. A single colony of *E. coli* DH5α was picked from the culture plate and inoculated into a 50 ml tube containing 5 ml of LB broth. This culture was incubated overnight at 37°C with shaking at 130 rpm.
2. From the overnight culture, 500 µl was transferred into a fresh 50 ml tube containing 5 ml of LB medium. The culture was incubated at 37°C on a shaker set to 250 rpm until it reached an optical density (OD_{600}) between 0.4 and 0.6.
3. A total of 1.5 ml from the secondary culture was aliquoted into two separate 1.5 ml microcentrifuge tubes.
4. The cells were centrifuged at 10,000 rpm for 1 minute at room temperature.
5. After centrifugation, the supernatant was completely removed, and the cell pellets were resuspended in 600 µl of pre-chilled, sterile transformation buffer.
6. The resuspended cells were kept on ice for 10–15 minutes.
7. Following the cold incubation, the cells were centrifuged again at 10,000 rpm for 1 minute at room temperature.

8. The supernatant was discarded, and the cell pellets were resuspended in 200 µl of pre-chilled sterile transformation buffer, making them ready for transformation

3.2.3. TRANSFORMATION OF PLASMID (PX459) INTO COMPETENT DH5α *E. COLI* CELLS

Transformation is a method through which bacterium incorporate genes from their surroundings. Some bacteria's have natural ability to take up foreign DNA. The bacteria are made competent to take up the foreign DNA in laboratories. DH5α *E. coli* cells can incorporate plasmids from their surroundings through transformation. The competent cells are given heat shock at 42 °C for about 90 seconds.

Requirements:

- Plasmid PX459 (Table 3.1.1.)
- E. coli competent cells [DH5α] (Table 3.1.2.)
- Appropriate Antibiotic (Ampicillin) (Table 3.1.3.)
- Luria-Bertani medium (LB) (Table 3.1.4.)
- Luria-Bertani agar (LA) (Table 3.1.5)
- Incubator
- LAF

Procedure:

1. 2µl of plasmid PX459 was added into 200 ul of DH5α competent cells prepared previously **3.2.2** in a microcentrifuge tube in LAF, then incubated in ice for 10 min.
2. Then the tubes were placed in a water bath that had been preheated to 42ºC for 90 seconds (heat shock).
3. The tubes were rapidly transferred on ice, and the cells were allowed to chill for 5 min.
4. Then 800 µl of LB medium was added into the tube and incubated for 90 min in an incubator at 37ºC on a shaker at >150 rpm.
5. The cells were then centrifuged at 10,000 rpm for 1 min in RT.
6. Approximately 700 µl of supernatant was discarded, remaining were re-suspended with pellet.
7. The remaining cell suspension was spread plated on LA plate containing an appropriate antibiotic i.e. Ampicillin in LAF.

8. The plates were incubated overnight in an incubator at 37ºC.
9. Transformed colonies (PX459 culture plate) was observed the next day and further procedures were carried out.

3.2.4. SINGLE COLONY STREAK PLATE

The single colony streak plate method is a microbiological technique used to isolate pure colonies of microorganisms from a mixed culture. In this project, a small amount (single colony) of PX459 bacterial sample was streaked from the culture plate across the surface of an LA plate containing ampicillin in a specific pattern, usually in three or four phases, using a sterile inoculating loop. As the loop is streaked across successive sections of the plate, the number of cells is diluted, leading to the formation of well-separated individual colonies. Each isolated colony originates from the chosen single microbial colony, making this method essential for obtaining pure cultures for further experimentation.

Requirements:

- Bacterial culture: colony from the PX459 culture plate.
- Luria-Bertani agar (LA) (Table 3.1.5)
- Appropriate Antibiotic (Ampicillin) (Table 3.1.3.)
- Bunsen Burner or Alcohol Lamp
- Incubator
- LAF

Procedure:

1. In LAF, a single colony was selected and picked from the PX459 culture plate using the sterile inoculating loop.
2. The sample was streaked (Quadrant streaking) on the LA plate containing ampicillin.
3. The plates were incubated overnight at 37ºC in an incubator.
4. Transformed colonies from a single colony were observed the next day.

3.2.5. BACTERIAL PLASMID ISOLATION (PX459)

Isolation of plasmid is carried out by alkaline lysis to break open the cell. The bacterial suspension is first treated with a resuspension buffer to degrade the cell wall. Then, the lysed

cell suspension is subjected to an alkaline lysis buffer comprising of Sodium Dodecyl Sulphate (SDS) (anionic surfactant) and a strong base like sodium hydroxide (NaOH). The anionic detergent cleaves the phospholipids of the membrane and denatures the proteins which play an important role in maintain the structure of the cell membrane. The NaOH's alkaline condition leads to denaturation of the plasmid and genomic DNA. Then a neutralization solution is added which contains the acidic potassium acetate that decreases the alkaline condition of the mixture and hence under the acidic condition the hydrogen bonds between complementary bases is restored. The small plasmid DNA anneals quickly owing to its circular and covalent nature whereas the huge genomic DNA cannot properly anneal. Thus, the plasmid remains in the suspension and the denatured genomic DNA and other proteins along with the SDS associate with each other by virtue of hydrophobic interactions and can be separated out by centrifugation as a pellet. The cleared lysate is then subjected to 100% ethanol to retrieve a concentrated plasmid solution.

Requirements:

- Overnight grown bacterial cells incorporated with PX459 taken from single colony streak plate.
- Plasmid DNA purification kit, [SLS RESEARCH PRIVATE LIMITED]
- Incubator
- Centrifuge
- LAF
- Nuclease free water

Procedure:

1. The bacterial cells incorporated with our plasmid (PX459) were cultured overnight in an incubator at 37ºC in a 50 ml tube containing 5ml LB medium with ampicillin on a shaker at 125rpm.
2. Next day, in LAF the cell suspension was transferred into a 1.5 ml microcentrifuge tube which was centrifuged at 10,000 rpm for 3 min in RT and the supernatant was discarded.
3. To the pelleted cells 250 µl of P1 buffer provided in kit was added and pellet was completely re-suspended by gentle mixing.
4. Then 250 µl of P2 buffer provided in kit was added and thorough mixing was carried out by inverting the tubes 6-10 times. The mixture was not allowed to be incubated for more than 5 min in RT.

5. Then, 350 µl of PN3 buffer provided in kit was added and mixing was carried out by inverting the tubes 6-10 times.
6. The mixture was then centrifuged at 10,000 rpm for 10 min at RT.
7. The SLS plasmid column was placed with a 2 ml collection tube and the supernatant was decanted into the column.
8. The column was centrifuged at 10,000 rpm for 1 min at RT and flow through was discarded.
9. This was followed by addition of 500 µl of PW1 (buffer provided in kit) and then the column was centrifuged at 10,000rpm for 1 min at RT. Flow through was discarded
10. The column was washed by adding 700 µl of PW2 buffer (Wash buffer with ethanol) (provided in kit) and centrifuged at 10,000 rpm for 1 min at RT. Flow through was discarded and column was placed in centrifuge for dry spin at 10,000 rpm for 30 seconds at RT.
11. The SLS RESEARCH PRIVATE LIMITED plasmid column was placed onto a new 1.5 ml microcentrifuge tube.
12. To elute the plasmid DNA a total of 50 µl of nuclease free water was added into the column where firstly 30 µl of NFW was added onto the column and incubated for 5 mins and centrifuged at 10,000 rpm for 1 min and then 20 µl of NFW was added onto the column, incubated for 5 min and then centrifuged at 10,000 rpm for 1 min at RT.
13. The small amount of eluted plasmid DNA was analysed on 1.5 % agarose gel in TAE buffer along with a 1 kb DNA marker.
14. The concentration of the isolated PX459 plasmid was measured using a Nanodrop.

3.2.6. AGAROSE GEL ELECTROPHORESIS

This technique is used to separate the DNA fragments by virtue of their molecular weight and size. In this procedure an electrical field is applied to move the negatively charged DNA, towards the positively charged electrode, through the agarose gel matrix. The smaller DNA fragments move faster than the larger fragments owing to their size. A DNA ladder, having different DNA fragments of known length, is also run alongside the gel to compare the size of the DNA sequence of interest. Ethidium bromide, an intercalating agent (intercalates into the major groove of DNA), is added to the agarose solution before the gel is cast. It fluoresces under Ultraviolet (UV) light and thus the gel can be viewed using an UV transilluminator or photographed in Gel-DOC apparatus.

Requirements:

- DNA samples
- Agarose powder [Sisco Research Laboratories Pvt. Ltd. (SRL)]
- Ethidium Bromide [Thermo, 20 mg/ml]
- DNA molecular weight marker (1 kb ladder)
- 10X TAE buffer [SRL]

Procedure:

1. The gel apparatus was cleaned using distilled water and wiped dry using clean tissue paper.
2. The gel tray was properly sealed on both the open ends with cello tape to avoid leakage and comb was inserted in place.
3. To prepare 1.5% gel, 30ml of TAE buffer was taken in a flask and 0.45 gm of agarose was added to it.
4. The mixture was then subjected to boiling in a microwave till the agarose completely dissolved in the buffer.
5. After cooling the mixture sufficiently, 2 µl of ethidium bromide (Et-Br) solution was added and mixed gently, to avoid any bubble formation.
6. The liquid mixture was then carefully poured into the gel tray and allowed to solidify.
7. After 20 min, the gel tray was placed in the electrophoresis tank and the comb was removed carefully.
8. 1X TAE buffer was poured into the tank such that the gel was submerged.
9. DNA samples were thoroughly mixed with appropriate volume of 1X gel loading dye and then were loaded into the gel.
10. To estimate the size of DNA, a DNA molecular weight marker (1 kb ladder) was also added into a well.
11. Electrophoresis was performed at 80V for appropriate required amount of time.

3.2.7. EcoRI RESTRICTION DIGESTION OF PX459 PLASMID (Confirmatory test)

Restriction digestion is the specific cleavage of the DNA using enzymes called as restriction enzymes such as EcoRI. EcoRI restriction enzymes cut a double stranded DNA, at two places, within a specific site referred to as the restriction recognition sequences. This test was

performed as a confirmatory test to check the presence of our desired plasmid PX459 in *E. coli* DH5α cells.

Requirements:

- Vector (plasmid PX459)
- 10X buffer. [Thermo Scientific]
- Nuclease free water.
- Water bath
- EcoRI restriction enzyme [Thermo Scientific]

ENZYME	RECOGNITION SEQUENCE	CUT SITES
EcoRI (5unit/µl)	5'…GAATTC…-3' 5'…CTTAAG…3'	i.5077 ii.5764

Table 3.2.7.1: List of restriction enzyme used.

Procedure:

1. Following reaction mixture was set up in a 100 µl microcentrifuge tube:

COMPONENTS	VOLUME	CONCENTRATON
Plasmid PX459 (205.8 ng/µl)	2 µl	411.6 ng
EcoRI enzyme (5unit/µl)	0.5 µl	2.5 unit
10x Buffer	1.5 µl	
NFW	16 µl	
Total Reaction volume	20 µl	

Table 3.2.7.2: Reaction set up for restriction digestion of PX459 with EcoRI.

2. The mixture was incubated in water bath at 37ºC for 2 hours.
3. A small amount of digested DNA was resolved on 1.5% agarose gel with 1X TAE buffer.

3.2.8 ANNEALING AND PHOSPHORYLATION OF OLIGOMERS

To prepare double-stranded DNA inserts for cloning, single stranded oligomers annealing and phosphorylation are essential processes. To enable hybridisation and the formation of double-stranded DNA, complementary single-stranded oligonucleotides are heated and then gradually cooled during the annealing step. After annealing, T4 polynucleotide kinase (PNK) is used to phosphorylate the oligos, adding phosphate groups to their 5' ends to facilitate effective ligation into plasmid vectors. The oligos are correctly paired and chemically ready for insertion into a digested plasmid PX459 backbone.

Requirements:

- BRCA2E18 top oligomer (100 µM)
- BRCA2E18 bottom oligomer (100 µM)
- 10x PCR (Taq) buffer
- T4 polynucleotide kinase (PNK) [Takara 10 U/µl]
- 10x PNK buffer [Takara]
- PCR machine [Rotor gene Q by Qiagen]
- Water bath

Procedure:

3.2.8.1. Annealing of oligomers

1. Following reaction mixture was set up in a 100 µl microcentrifuge tube:

COMPONENTS	VOLUME AND CONCENTRATION
BRCA2E18 Top oligomer (100 µM) (5’ CACCGCTTCATGGAGCAGAACTGGT 3’)	2.5 µl (250µM)
BRCA2E18 Bottom oligomer (100 µM) (5’ AAACACCAGTTCTGCTCCATGAAGC 3’)	2.5 µl (250µM)
10x PCR (Taq) buffer	5 µl
NFW	40 µl
Total Reaction volume	50 µl

Table 3.2.8.1a: Reaction set up for annealing through gradient temperature.

2. The reaction mixture was then subjected to annealing through gradient temperature as mentioned below using a PCR machine:

Step No.	Temperature (ºC)	Time (minutes)
1	95	2
2	90	5
3	85	5
4	80	5
5	75	5
6	70	5
7	65	5
8	60	5
9	55	5
10	50	5
11	45	5
12	40	5
13	35	5
14	25	5
15	4	∞

Table 3.2.8.1b: Conditions for annealing through gradient temperature.

3.2.8.2. Phosphorylation of oligomers

1. Following reaction mixture was set up in a 100 µl microcentrifuge tube:

COMPONENTS	VOLUME AND CONCENTRATION
Annealed Oligomer (5 µM)	44 µl (220 µM)
T4 polynucleotide kinase (PNK)	1 µl
10X PNK buffer	5 µl
Total Reaction volume	50 µl

Table 3.2.8.2: Reaction set up for phosphorylation of oligomers.

2. The reaction mixture was incubated at 37ºC for 2 hours in a water bath.

3. BRCA2E18 oligomers both top and bottom were annealed and phosphorylated. Annealed oligomers are ready to be used as the insert for ligation into the plasmid PX459 and was named as **BRCA2E18 AS**.

3.2.9. BBSI RESTRICTION DIGESTION OF PX459 PLASMID

The specific cleavage of DNA by restriction enzymes, such as BbsI, is known as restriction digestion. Within a particular site known as the restriction recognition sequences, restriction enzymes cut double-stranded DNA twice. Palindromes typically make up such sequences. While some restriction enzymes produce blunt ends, others make incisions that result in staggered ends, which aid in future effective ligation. BbsI is a hexacutter type IIs restriction enzyme which produces sticky ends that aid in the cloning of desired DNA inserts, such as the sticky-ended BRCA2E18 AS.

Requirements:

- Vector (plasmid PX459)
- 10X buffer. [Thermo Scientific]
- Nuclease free water.
- Water bath
- BbsI restriction enzyme [Thermo Scientific]

ENZYME	RECOGNITION SEQUENCE	CUT SITES
BbsI (10unit/µl)	5'… GAAGACNN…3' 5'…NNCAGAAG…3'	i.8977 ii.8999

Table 3.2.9.1: List of restriction enzyme used.

Procedure:

1. Following reaction mixture was set up 100 in a µl microcentrifuge tube:

COMPONENTS	VOLUME	CONCENTRATION
Plasmid PX459 (205.8 ng/µl)	9.8 µl	2016.84 ng
BbsI enzyme	1 µl	10 unit

10x BbsI buffer	5 µl	
NFW	34.2 µl	
Total Reaction volume	50 µl	

Table 3.2.9.2: Reaction set up for restriction digestion of PX459 with BbsI

2. The mixture was then incubated in water bath at 37ºC for 2 hours.
3. A small amount of digested DNA (PX459) was resolved on 1.5% agarose gel with 1X TAE buffer.
4. Rest of the digested plasmid was then purified using PCR purification kit (SLS), where the digested plasmid was treated with PW1 buffer in a spin column and centrifuged at 10,000rpm for 1 min at RT.
5. Flow through was discarded, and PW2 buffer was added into spin column and centrifuged at 10,000rpm for 1 min at RT.
6. Flow through was discarded, after an empty spin the digested plasmid was eluted in 50 µl of NFW.

3.2.10. LIGATION OF INSERT BRCA2E18 AS WITH BbsI DIGESTED VECTOR PX459

Ligation is catalysed by the enzyme known as DNA ligase. It catalyses the formation of a phosphodiester bond between 3'-hydroxyl and 5'-phosphate groups of the DNA backbone. DNA ligation is a normal process in cell and is a part of repair machinery to repair nicks in single or both the strands of DNA. In this study, ligation procedure is used to join the BRCA2E18 AS and BbsI digested plasmid PX459. Before setting up the reaction mixture for ligation, the Bbs1 digested plasmid is purified to remove any sort of contamination.

Requirements:

- Vector (BbsI digested and purified plasmid PX459)
- Insert (BRCA2E18 AS)
- T4 DNA ligase [SRL, 1 Weiss U/µl]
- Ligation Buffer [SRL]
- Nuclease free water
- LAF

Procedure:

1. The following ligation mixture was set up in LAF for ligation in a 100 µl microcentrifuge tube:

COMPONENTS	VOLUME	CONCENTRATION
Vector DNA (16 ng/µl)	4 µl	64 ng
Insert DNA (4.4µM)	1 µl	4.4 µM
T4 DNA Ligase	1 µl	
Ligation Buffer	1 µl	
NFW	4 µl	
Total Reaction volume	11 µl	

Table 3.2.10.1: Reaction set up ligation of vector and insert.

2. The ligation mixture was incubated at RT for 4 hours.
3. DH5α (*E. coli)* competent cells were prepared same as **3.2.2.**

3.2.11. TRANSFORMATION OF RECOMBINANT PLASMID INTO THE COMPETENT *E. COLI* CELLS (DH5α)

Requirements:

- Recombinant Plasmid (Insert + Vector)
- E. coli competent cells [DH5α] (Table 3.1.2.)
- Appropriate Antibiotic (Ampicillin) (Table 3.1.3.)
- Luria-Bertani medium (LB) (Table 3.1.4.)
- Luria-Bertani agar (LA) (Table 3.1.5)
- Incubator
- LAF

Procedure:

1. In LAF 11µl of ligation mixture (recombinant plasmid) was added into 200ul of *E. coli* competent cells in a 500 µl microcentrifuge tube, then incubated in ice for 10 min.
2. Then the tubes were placed in a water bath that had been preheated to 42ºC for 90 seconds (heat shock).
3. The tubes were rapidly transferred on ice and allowed the cells to chill for 5 min.
4. 800 µl of LB medium was added into the tube and incubated in an incubator for 90 min at 37ºC on a shaker at >150 rpm.
5. The cells were then centrifuged at 10,000 rpm for 1 min in RT.
6. Approximately 700 µl of supernatant was discarded, remaining were re-suspended with pellet.
7. The re-suspended cell suspension was spread plated on LA plate containing an appropriate antibiotic i.e. Ampicillin in LAF.
8. The plates were then incubated overnight in an incubator at 37ºC.
9. Transformed colonies containing our recombinant plasmid was observed next day and further procedures were carried out.

3.2.12. REPLICA PLATING OF TRANSFORMED COLONIES

A microbiological method for moving bacterial colonies from one agar plate to another in the same spatial arrangement is called "replica plating of transformed colonies." It is frequently used to screen for phenotypes, like antibiotic resistance. By contrasting growth patterns on selective media, replica plating enables the identification of clones that have successfully incorporated the recombinant plasmid in the case of transformed colonies. Colonies from the recombinant plasmid master plate are streaked onto fresh plates using a sterile inoculation loop, guaranteeing uniform colony positioning for simple comparison.

Requirements:

- Colonies of transformed bacterial cells incorporated with recombinant plasmid master plate.
- Luria-Bertani Agar (LA) Plate containing ampicillin.
- Sterile inoculating Loop.
- Incubator
- LAF

Procedure:

1. Using a marker, nine quadrants were marked starting from C1 – C9 on the back side of the Luria-Bertani agar plate.
2. In LAF, a total of nine colonies from the transformed colonies incorporated with the recombinant plasmid master plate were selected and streaked onto each quadrant respectively.
3. The plate was incubated overnight in the incubator at 37ºC.
4. Selected transformed colonies containing our recombinant plasmid was observed next day and further procedures were carried out.

3.2.13. COLONY PCR

A high throughput technique for detecting whether insert DNA (BRCA2E18 AS) is present in the plasmid (PX459) is colony PCR. Colony PCR is used to ascertain whether the insert is orientated correctly. A small portion of a colony is picked and directly used as the template in a PCR reaction without prior DNA purification. The cells are lysed by heat or through the PCR process itself, releasing DNA for amplification. Specific primers are used to amplify target regions, confirming the presence or orientation of the insert. This technique is commonly used after transformation to quickly identify positive clones. Using gel electrophoresis comparison of the bands produced is done with a DNA marker, the presence or absence of the PCR amplicons is ascertained.

Requirements:

- Template - Colonies from replica plate. (C1, C2, C3 and C4)
- 10x PCR buffer
- DNTP
- Taq DNA polymerase
- NFW
- Thermal Cycler
- PCR Cabinet
- Primers (Insert Specific)

PRIMERS	SEQUENCE	CONCENTRATION
U6 Forward	5’ GCCTATTTCCCATGATTCC 3	10 µM

BRCA2E18 bottom oligomer	5' AAACACCAGTTCTGCTCCATGAAGC 3'	10 µM

Table 3.2.13.1: List of primers used in colony PCR.

Procedure:

1. Inside the PCR cabinet he following components were added in a 1.5 ml microcentrifuge tube.

COMPONENTS	1 REACTION (20µl)	6 REACTIONS (Master Mix)
NFW	14.75 µl	88.5 µl
10x PCR buffer	2 µl	12 µl
DNTP	2 µl	12 µl
U6 Forward Primer	0.5 µl	3 µl
BRCA2E18 Bottom Oligo	0.5 µl	3 µl
Taq	0.25 µl	1.5 µl

Table 3.2.13.2: Reaction set up for colony PCR

2. 20 µl of master mix was taken in 5 PCR tubes each.
3. Using different tips, C1, C2, C3 and C4 colonies were mixed into four different PCR tubes respectively.
4. Last PCR tube was kept as a control where no colonies were mixed.
5. The following PCR conditions were utilized for PCR amplification.

STEP No.	STEP	TEMPRETURE	TIME
I	Initial denaturation	94ºC	5 min
II	Denaturation	94 ºC	30 sec
III	Annealing	60 ºC	30 sec
IV	Extension	72 ºC	48 sec
V	Final extension	72 ºC	5 min
VI	Hold	25 ºC	1 min

Table 3.2.13.3: Conditions used for colony PCR

Step II – IV were repeated for 30 cycles

6. PCR products were resolved on 1.5% agarose gel in 1X TAE buffer.

3.2.14. RECOMBINANT PLASMID ISOLATION

The recombinant plasmids having the desired insert (BRCA2E18 AS) can be extracted from transformed bacterial cells using a technique called bacterial plasmid isolation **3.2.5**. Bacteria containing the plasmid are usually cultured in LB medium, and then the cells are broken open by alkaline lysis, which denatures the proteins and chromosomal DNA. After that, the plasmid DNA is purified by spin-column techniques. For subsequent uses like End Point PCR (primers specific to the vector) to confirm the presence of recombinant plasmid and sanger sequencing, this isolated recombinant plasmid can be employed.

Requirements:

- Overnight grown bacterial cells incorporated with recombinant plasmid. (2 colonies chosen from the colonies used in colony PCR)
- Incubator
- Plasmid DNA purification kit, [SLS RESEARCH PRIVATE LIMITED]
- Centrifuge
- Nanodrop
- Nuclease free water

Procedure:

1. The bacterial cells from 2 colonies C1 and C2 were grown overnight in an incubator at 37ºC in 5ml LB medium with ampicillin.
2. Next day, the cell suspension of the colonies was centrifuged in 2 different microcentrifuge tubes (1.5 ml) respectively at 10,000 rpm for 3 min in RT and the supernatant was discarded.
3. To the pelleted cells 250 µl of P1 buffer provided in kit was added and pellet was completely re-suspended by gentle mixing.
4. Then 250 µl of P2 buffer was added provided in kit and thorough mixing was carried out by inverting the tubes 6-10 times. The mixture was not allowed to be incubated for more than 5 min in RT.

5. Then, 350 µl of PN3 buffer was added provided in kit and mixing was carried out by inverting the tubes 6-10 times.
6. The mixture was centrifuged at 10,000 rpm for 10 min at RT.
7. The SLS plasmid column was placed with a 2 ml collection tube and the supernatant was decanted into the column.
8. The column was centrifuged at 10,000 rpm for 1 min at RT and flow through was discarded.
9. This was followed by addition of 500 µl of PW1 buffer provided in kit and then the column was centrifuged at 10,000rpm for 1 min at RT. Flow through was discarded
10. The column was washed by adding 700 µl of PW2 buffer (Wash buffer with ethanol) provided in kit and centrifuged at 10,000 rpm for 1 min. Flow through was discarded and column was placed in centrifuge for dry spin at 10,000 rpm for 30 seconds at RT.
11. The SLS RESEARCH PRIVATE LIMITED plasmid column was placed onto a new 1.5 ml microcentrifuge tube.
12. To elute the recombinant plasmid DNA for both the colonies a total of 50 µl of nuclease free water was added into the column where firstly 30 µl of NFW was added onto the column and incubated for 5 mins and centrifuged at 10,000 rpm for 1 min and then 20 µl of NFW was added onto the column, incubated for 5 min and then centrifuged at 10,000 rpm for 1 min at RT.
13. The eluted recombinant plasmid DNA of colony C1 and C2 concentration was measured using a nanodrop.
14. After then, end-point PCR conducted to confirm successful ligation using a designed primers specific to the vectors.

3.2.15. PRIMER DESIGNING

In the end-point PCR (Polymerase Chain Reaction) process, primer design is an essential step that entails producing brief single-stranded DNA sequences complementary to the target DNA region. These primers attach to the template DNA and give DNA polymerase a place to start synthesising new DNA. They are typically 18–25 nucleotides long. Effective primer design ensures specificity, optimal melting temperature (Tm), balanced GC content (40–60%), and minimal secondary structures like hairpins or dimers. Primers are frequently designed using software programs like NCBI Primer-BLAST to ensure precise and effective amplification. In this project the primers that are designed are specific to the vector (PX459).

Requirements:

- Internet access
- Target DNA sequence of pSpCas9(BB)-2A-Puro (PX459) V2.0 (in FASTA format)
- Primer design software (NCBI Primer-BLAST)
- Oligo analysis tool (SnapGene)

Procedure:

1. Retrieval of PX459 plasmid sequence.
   The full plasmid sequence was retrieved in **FASTA (. fasta)** format from the Addgene website: https://www.addgene.org/62988/. The SnapGene file downloaded and saved it locally.
2. The sequenced was opened in SnapGene and the target region i.e. 150bp away from the BbsI sites was identified and copied for designing primers.
3. The copied target DNA sequence was pasted in the NCBI Primer-BLAST: https://www.ncbi.nlm.nih.gov/tools/primer-blast/ tool template box.
4. The parameters for designing the primers were adjusted.
5. **"**Get Primers**"** was clicked to generate a list of suggested primer pairs.
6. The primer pairs were reviewed, and primer pair 3 was chosen.
7. Only the reverse primer was copied and pasted in SnapGene plasmid PX459 file, the binding of primer to the plasmid PX459 was visualised and the amplicon size was determined which will be produced after end point PCR of the isolated recombinant plasmid.
8. The reverse primer was named as U6 reverse primer and was ordered.

3.2.16. END POINT PCR (POLYMERASE CHAIN REACTION)

End-point PCR is a method for amplifying the target DNA sequences and can be frequently used to verify the integrity and presence of recombinant plasmid. Primers in this situation are made to target areas that surround the inserted gene or a junction where the vector and insert meet. 1.5% Agarose gel electrophoresis is used to analyse the PCR product following amplification. The successful cloning and insertion of the desired DNA fragment into the plasmid is indicated by the presence of a band of the expected size, which validates the plasmid's recombinant nature.

Requirements:

- Template – Plasmid DNA of the colonies C1 and C2.
- 10x PCR buffer
- DNTP
- Taq DNA polymerase
- NFW
- Thermal Cycler
- PCR Cabinet
- Primers (Vector Specific)

PRIMERS	SEQUENCE	CONCENTRATION
U6 Forward	5' GCCTATTTCCCATGATTCC 3	10 µM
U6 Reverse	5' CACGCGCTAAAAACGGACTA 3'	10 µM

Table 3.2.16.1: List of primers used in end point PCR.

Procedure:

1. Inside the PCR cabinet the following components were added in a 1.5 ml microcentrifuge tube:

COMPONENTS	1 REACTION (20µl)	4 REACTIONS (Master Mix)
NFW	14.75 µl	59 µl
10x PCR buffer	2 µl	8 µl
DNTP	2 µl	8 µl
U6 Forward Primer	0.5 µl	2 µl
U6 Reverse Primer	0.5 µl	2 µl
Taq	0.25 µl	1 µl

Table 3.2.16.2: Reaction set up for end point PCR.

2. 20 µl of master mix was taken in 3 PCR tubes each.
3. 2 µl of template was added into 2 different PCR tubes.
4. Last PCR tube was kept as a control where no colonies were mixed.
5. The following PCR conditions were utilized for PCR amplification:

STEP No.	STEP	TEMPRETURE	TIME
I	Initial denaturation	95 ºC	5 min
II	Denaturation	95 ºC	30 sec
III	Annealing	56 ºC	30 sec
IV	Extension	72 ºC	48 sec
V	Final extension	72 ºC	5 min
VI	Hold	25 ºC	1 min

Table 3.2.16.3: Conditions used for end point PCR

Step II – IV were repeated for 30 cycles

6. PCR products were resolved on 1.5% agarose gel in 1X TAE buffer.

3.2.17. SANGER SEQUENCING

Sanger sequencing is a key technique used in the thesis project to verify the accuracy of recombinant plasmid constructs. After cloning and transformation, selected plasmid DNA samples are sequenced using sanger sequencing to confirm the correct insertion and orientation of the target gene. This method provides high-quality, base-level confirmation, ensuring that no mutations or errors occurred during amplification or ligation steps. The sequencing results are aligned with the reference sequence to validate the integrity of the recombinant plasmid, supporting the reliability of downstream experiments.

Requirements:

- Plasmid DNA - High-quality, contamination-free DNA extracted from transformed colonies C1 and C2.
- Sequencing Primer – (U6 Forward primer 10µM)

Procedure:

1. 20 µl of plasmid DNA (2µg) was aliquoted into a 1.5 ml microcentrifuge tube for both recombinant plasmids.
2. 10 µl of U6 forward primer (10µM) was aliquoted into a 1.5 ml microcentrifuge tube.
3. Both aliquoted primer and plasmid DNA was sent for sanger sequencing
4. The electropherogram of both plasmids were analysed carefully.

4. RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

4.1. sgRNA DESIGNING

The single guide RNA (sgRNA) was designed to target Exon 18 of the BRCA2 gene, a region of interest due to its role in DNA repair and its implication in cancer when mutated. The reference sequence for BRCA2 was retrieved from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/gene/675) to ensure accuracy and relevance to the human genome.

Candidates of sgRNA sequences targeting the BRCA 2 gene were generated using the CHOPCHOP (https://chopchop.cbu.uib.no/) online tool, which provides potential CRISPR/Cas9 target sites based on user-defined parameters such as target gene, exon region, and PAM sequence specificity.

From the list of sgRNA candidates, the top-ranking sgRNA sequence was chosen as the target sequence i.e. "5'- CTTCATGGAGCAGAACTGGTGGG -3'". It was selected based on several factors including high on-target score, low predicted off-target effects, and its location within the coding region of exon 18.

The following parameters were used to select the most optimal sgRNA:

- GC content between 40–60% for stable binding and transcription. GC content of our target sequence is 50 %.
- High on-target efficacy score (>50%). The target sequence has a 64.78 % efficiency.
- Minimal predicted off-target effects. The chosen target sequence has 0 off-target.
- Minimal Self- complementarity. The chosen target sequence has 0 self-complementarity
- Proximity to functional exons or critical protein domains of BRCA2

The top-ranking sgRNA sequence was selected and converted into a pair of oligonucleotides for cloning into the CRISPR-Cas9 vector (pSpCas9(BB)-2A-Puro (PX459)). Overhangs compatible with BbsI restriction enzyme sites were added.

- BRCA2E18 Top Oligomer: 5'- **CACCG**CTTCATGGAGCAGAACTGGT -3' [25-nt sgRNA sequence]
- BRCA2E18 Bot Oligomer: 5'-**AAAC**ACCAGTTCTGCTCCATGAAGC -3' [25-nt sgRNA sequence]

Into the top strand (5′-CACCG...-3′) overhang was added and to the bottom strand (5′-AAAC...-3′) overhang was added.

The inclusion of specific 5 bp overhangs CACCG on the top strand and 4 bp AAAC on the bottom strand facilitates directional cloning and ensures proper orientation within the plasmid.

4.2. PREPARATION OF COMPETENT CELLS (DH5α)

The calcium chloride technique, which changes the porousness of the membrane, to make cells permeable to foreign DNA, was successfully used to create competent *E. coli* DH5α cells. The cells were prepared as mentioned in the procedure **3.2.2** and cultured until they reached the mid-log phase ($OD_{600} \approx 0.4–0.6$), which is when transformation is most likely to occur. To foster competence, the cell pellets were centrifuged, then gently resuspended in a 200 µl of transformation buffer. The cells appeared creamy and uniform, indicating good viability and proper handling throughout the protocol.

The competent cells were stored in 4ºC to avoid any loss of competency. To evaluate competency, a transformation efficiency test was performed using the PX459 plasmid. The resulting number of colonies on LA-ampicillin plates suggested a transformation efficiency within the expected range for chemically competent DH5α cells.

4.3 TRANSFORMATION OF PLASMID PX459

The transformation of *E. coli* DH5α competent cells with the plasmid PX459 (Cas9 expression vector) was successfully performed using the heat-shock method as mentioned in the procedure of **3.2.3** i.e. 45ºC for 90 sec. Two LB agar plates containing ampicillin (100 µg/mL) were used: one with the transformed cells (experimental plate) and one with untransformed competent cells (negative control).

Fig 4.3.1: Positive Test (PX459)

Fig 4.3.2: Negative Control (PX459)

On the experimental plate (left), numerous well-isolated colonies were observed, indicating successful uptake of the pX459 plasmid, which confers ampicillin resistance. The high colony count suggests that the competent DH5α cells had good transformation efficiency, and the plasmid was successfully maintained and expressed.

In contrast, the negative control plate (right), which contained competent DH5α cells without plasmid DNA, showed no colony growth, confirming that the ampicillin resistance observed on the experimental plate was due to successful transformation with PX459. This also rules out contamination or inherent resistance of the competent cells.

The results validate both the efficiency of the competent cell preparation and the successful incorporation of the PX459 plasmid, which can now be used as aan expression vector for downstream applications such as cloning of our BRCA2E18 insert for CRISPR/Cas9-mediated genome editing.

4.4. STREAK PLATING OF SINGLE COLONY

A single colony from the transformation plate containing the plasmid DNA (PX459) was streaked on an LB agar plate supplemented with ampicillin to isolate a pure bacterial colony. The purpose of this streak plate was to obtain well-isolated colonies derived from a single transformed cell for downstream applications such as plasmid extraction.

Fig 4.4: Single colony streak plate (PX459)

As shown in the plate image, distinct and well-separated colonies appeared along the streak lines, particularly in the final streak region. This indicates successful isolation of single colonies and confirms that the transformed *E. coli* cells (DH5α) retained the plasmid containing the ampicillin resistance gene. The growth pattern also suggests good viability and proper handling during streaking.

No contamination or unexpected growth was observed, supporting the purity of the colony used. The presence of discrete colonies derived from a single clone ensures genetic consistency in subsequent experiments. Overall, the streak plate technique effectively yielded isolated colonies, providing a reliable source of monoclonal culture for further procedures to be carried out i.e. making of an expression vector which will be ligated with the BRCA2E18 insert.

4.5 BACTERIAL PLASMID ISOLATION (PX459)

The Plasmid DNA purification kit [SLS RESEARCH PRIVATE LIMITED], which was previously mentioned in the procedure of 3.2.5, was used to successfully isolate Plasmid DNA PX459 from E. coli DH5α cells using a standard alkaline lysis method. The quality and presence of the plasmid DNA were assessed by resolving it in 1.5% agarose gel electrophoresis.

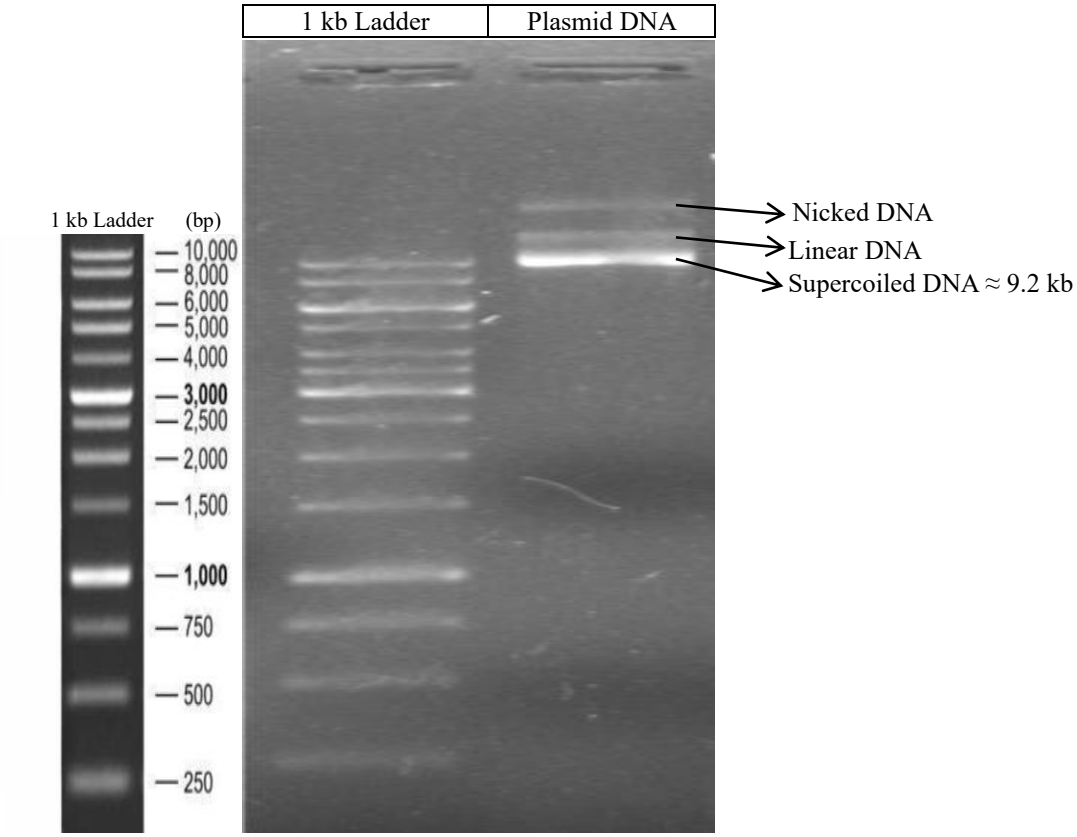


Fig 4.5: Gel Electrophoresis of isolated plasmid PX459

Lane 1: 1 kb DNA ladder (molecular weight marker)

Lane 2: PX459 plasmid DNA

As seen in the gel image, the plasmid lane shows multiple distinct bands. The most prominent band corresponds to approximately 9.2 kb, which matches the expected size of the PX459 plasmid. In contrast, the 1 kb DNA ladder run alongside provides clear molecular weight reference markers, confirming the approximate size of the plasmid. Additional bands observed above the main band likely represent different conformations of the plasmid DNA, such as nicked or linearized forms, which is typical for plasmid preparations.

The concentration of the isolated plasmid PX459 was measured using a Nanodrop which turned out to be 205.8 ng/µl. The A260/280 ratio was 1.81. The presence of sharp and distinct bands without smearing indicates that the plasmid DNA was successfully isolated with minimal degradation and without any RNA contamination. This result confirms the successful transformation and replication of the PX459 plasmid in *E. coli* DH5α cells. The plasmid was isolated was very efficiently, providing good-quality DNA for downstream applications such as restriction digestion, cloning, PCR and sanger sequencing

4.6. RESTRICTION DIGESTION OF PLASMID PX459 USING EcoRI RESTRICTION ENDONUCLEASE (Confirmatory Test)

To confirm the identity and integrity of the isolated PX459 plasmid from DH5α cells, a restriction digestion was performed using EcoRI restriction endonuclease as detailed in the reaction setup **Table 3.2.7.2.** EcoRI a well-characterized restriction enzyme that recognizes the 5'…GAATTC…3' sequence. EcoRI sequences usually comprise of palindromes.

Eco1 restriction enzymes create incisions in such a way that it produces staggered ends. The total restriction recognition sites present in the PX459 is two and are located at 5077 and 5764 position. EcoRI is a hexacutter restriction endonuclease which yields sticky ends and cuts at 2 different sites when incubated at 37ºC. The digested plasmid was analysed by 1.5 % agarose gel electrophoresis alongside uncut plasmid and a 1 kb DNA ladder for size reference.

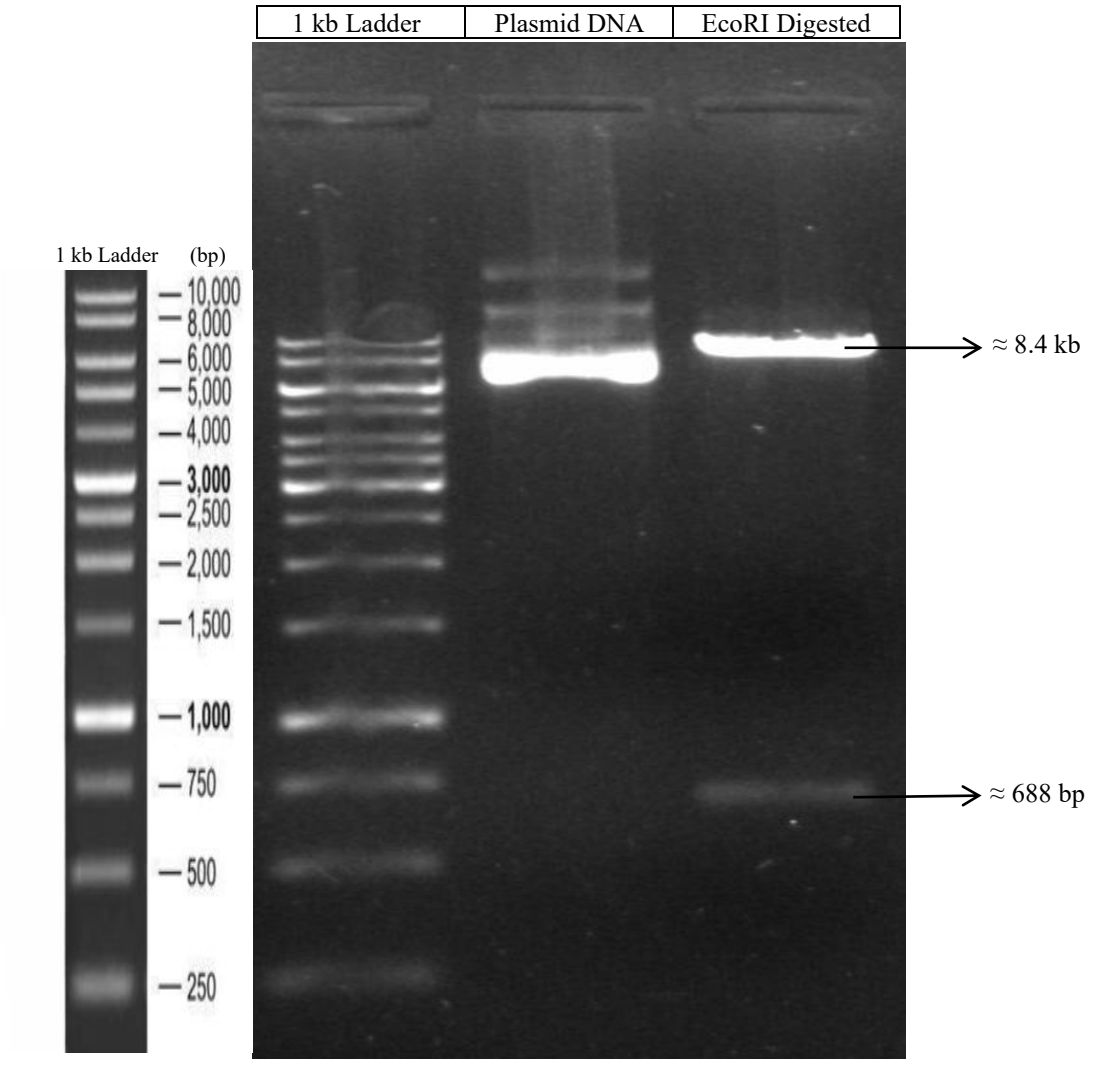


Fig 4.6: Gel Electrophoresis of digested PX459 with EcoR1 Restriction enzyme

Lane 1: 1 kb DNA ladder (molecular weight marker)
Lane 2: Uncut PX459 plasmid DNA
Lane 3: Plasmid PX459 DNA digested with EcoRI Restriction endonuclease

The uncut plasmid lane displayed multiple bands corresponding to different plasmid conformations nicked and linear forms, which is typical for undigested plasmid DNA. This pattern is consistent, as previously observed (~ 9.2 kb).

In contrast, the EcoRI digested plasmid showed 2 distinct band near the expected size of 8.4kb and 688 bp, consistent with complete linearization of the plasmid by EcoRI. The disappearance of the multiple conformational bands seen in the uncut sample and the appearance of two linear band confirms that:

- The plasmid was successfully digested, verifying the integrity and identity of the plasmid PX459.
- The DNA is of good quality and is free from inhibitors that could interfere with enzymatic activity.

This digestion serves as a confirmatory test, ensuring that the plasmid isolated is indeed PX459 and that it retains the correct sequence and structure necessary for downstream applications regarding the project.

4.7. ANNEALING AND PHOSPHORYLATION OF BRCA2E18 OLIGOMERS

To generate a double-stranded guide RNA (sgRNA) insert targeting exon 18 of the BRCA2 gene, complementary top and bottom strand oligomers were designed and synthesized. The BRCA2E18 Top Oligomer (5′CACCGCTTCATGGAGCAGAACTGGT-3′) and the BRCA2E18 Bottom oligomer (5′- AAACACCAGTTCTGCTCCATGAAGC -3′) were chosen based on the target sequence identified through CHOPCHOP, which ensures high specificity and minimal off-target activity.

The single stranded oligomers were subjected to a two-step process to finally construct our double stranded BRCA2E18 insert:

1. Annealing
2. Phosphorylation

Annealing of the oligomers was accomplished after the set up detailed in **Table 3.1.8.1a** by binding the two complementary single-stranded DNA oligomers (BRCA2E18 Top oligomer 100 µM and BRCA2E18 Bottom oligomer 100µM) together to form double-stranded DNA through hydrogen bonding between complementary base pairs. Successful annealing was

indicated by the formation of double-stranded oligonucleotides with compatible overhangs for BbsI cloning sites present in the PX459 vector. It was achieved through gradient temperature using a PCR machine **Table 3.2.8.1b**. The concentration of annealed duplex in 50 µL reaction was 5 µM.

Once both the single stranded oligomer is annealed to form a double stranded DNA, phosphorylation of the duplex is done using T4 Polynucleotide Kinase (PNK) to add 5′ phosphate groups as detailed in **Table 3.2.8.2**, which are necessary for subsequent ligation into the PX459 vector. The final concentration after phosphorylation was 4.4 µM in 50 µl.

The following is the double stranded sgRNA sequence after annealing and phosphorylation which will be our desired insert which was named as BRCA2E18 AS:

5’ **CACCG**CTTCATGGAGCAGAACTGGT 3’
CGAAGTACCTCGTCTTGACCA**CAAA** 5’

This step is critical for the CRISPR/Cas9 gene editing workflow, as it ensures the designed sgRNA can be properly transcribed and guide the Cas9 endonuclease to the genomic target. The resulting duplexed, phosphorylated oligos were used for ligation into the linearized PX459 vector for transformation into *E. coli* DH5α.

4.8. RESTRICTION DIGESTION OF PX459 PLASMID USING BbsI RESTRICTION ENDONUCLEASE

The goal of digesting the PX459 plasmid with BbsI as detailed in the reaction setup **Table 3.2.9.2.** was to create compatible sticky ends for cloning the BRCA2E18 AS insert into the plasmid backbone using Target Sequence Cloning Protocol, ZhangLab. BbsI is a Type IIS restriction enzyme that cuts DNA outside of its recognition sequence i.e. 5’…GAAGACNN…3’ sequence. BbsI cut site sequences usually comprise of palindromes. The PX459 plasmid was successfully digested with the BbsI restriction enzyme. BbsI is a hexacutter restriction endonuclease which yields sticky ends and cuts at 2 different sites when incubated at 37ºC. BbsI restriction enzymes create incisions at the restriction recognition sites present in the PX459 located at 8977 and 8999 position in such a way that it produces staggered ends onto which our BRCA2E18 AS insert will be ligated. The digested plasmid was analysed by 1.5 % agarose gel electrophoresis alongside uncut plasmid and a 1 kb DNA ladder for size reference.

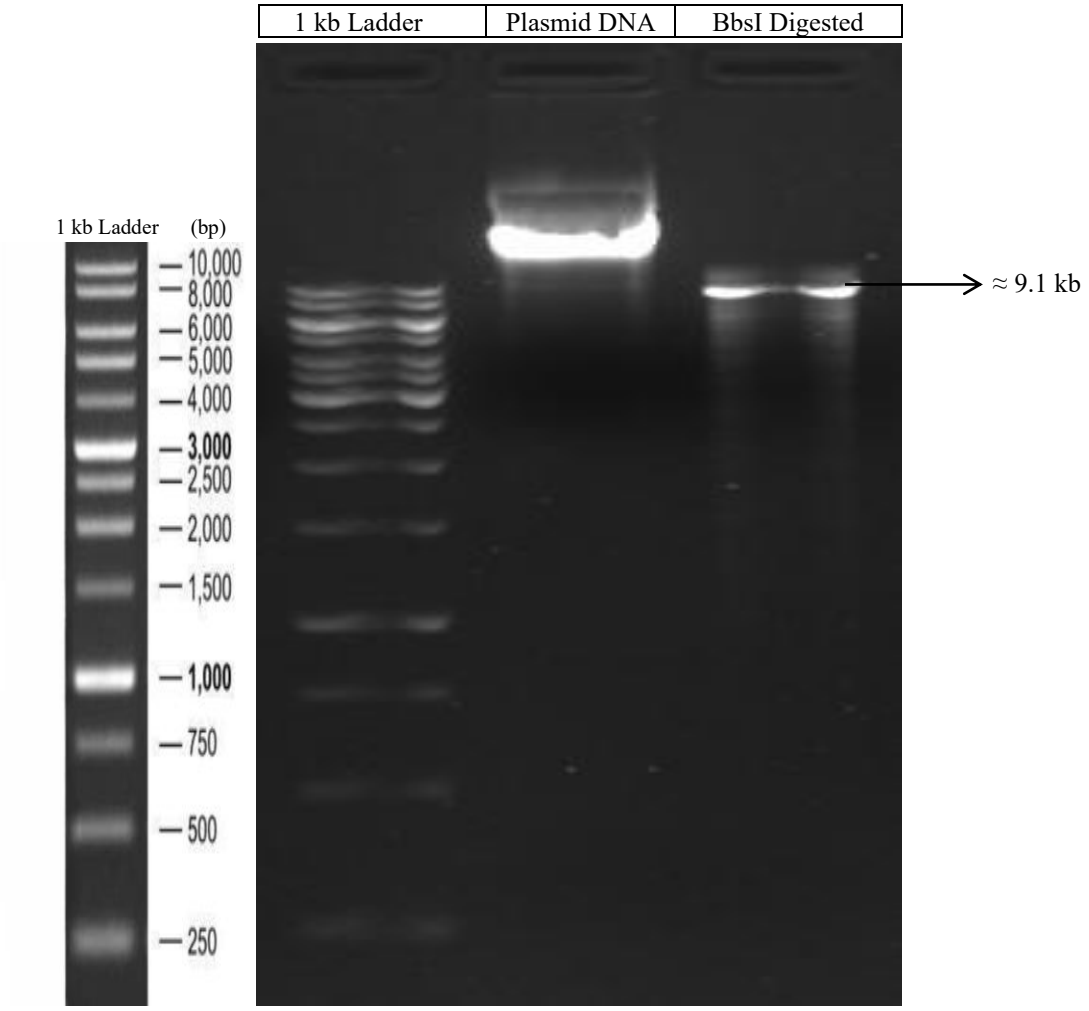


Fig 4.8: Gel Electrophoresis of digested PX459 with BbsI Restriction enzyme

Lane 1: 1 kb DNA ladder (molecular weight marker)
Lane 2: Uncut PX459 plasmid DNA
Lane 3: Plasmid PX459 DNA digested with BbsI Restriction endonuclease
This agarose gel image demonstrates the successful restriction digestion of the PX459 plasmid by the BbsI enzyme. The uncut plasmid lane shows a prominent band corresponding to the supercoiled form, along with faint bands representing the possibly nicked forms, which is typical of uncut plasmids.

In contrast, the BbsI-digested lane shows single clear distant band near the expected size 9.1 kb when compared with the 1 kb DNA ladder, indicating linearization or complete digestion of the plasmid. The clarity and sharpness of the band in the digested lane suggest efficient enzyme activity and minimal star activity or degradation. Concentration of digested plasmid was calculated to be 40.33 ng/µl (2016.84ng ÷ 50).

The linearized plasmid runs faster compared to the nicked form, confirming that BbsI enzyme successfully cleaved the specific recognition sites in the PX459 vector. PX459 contains BbsI sites flanking the gRNA cloning site, and digestion exposes overhangs compatible with the phosphorylated and annealed BRCA2E18 oligonucleotides. This digestion step is critical in preparing the plasmid for the insertion of target-specific oligonucleotides using Target Sequence Cloning Protocol, ZhangLab.

4.9. LIGATION AND TRANSFORMATION OF CLONES

Before the ligation of the BbsI digested PX459 vector with our double stranded BARC2E18 AS from **4.7,** the digested plasmid was purified to remove any sort of contamination and to isolate pure BbsI digested plasmid for ligation. PCR purification kit (SLS) was used to purify the digested plasmid

Before the purification the concentration of the BbsI digested PX459 plasmid was 40 ng/µl (Total = 1600ng in 40µL), but after purification the expected concentration of BbsI digested PX459 plasmid was calculated to be 16 ng/µl (Total = 800 ng in 50µl). After purification it is considered that half of the DNA will be lost during the process. Before purification 1600 ng of digested DNA was present in 40 µl reaction, which got reduced to 800 ng after purification when eluted in 50 µl NFW thus giving us the concentration of the purified BbsI digested plasmid as 16 ng/µl.

The insert (BRCA2E18 AS) was successfully ligated into the PX459 plasmid vector using a T4 ligase-mediated reaction, as detailed in the reaction setup **Table 3.2.10.1**. The ligation mixture was transformed into competent DH5α E. coli cells. Following transformation and plating on LB agar containing ampicillin, numerous colonies were observed on the experimental plate, while no colonies appeared on the negative control plate (vector without insert), as shown in the images. The presence of colonies on selective media indicates successful uptake of recombinant plasmids by the host cells. Further confirmation of positive clones was performed using colony PCR.

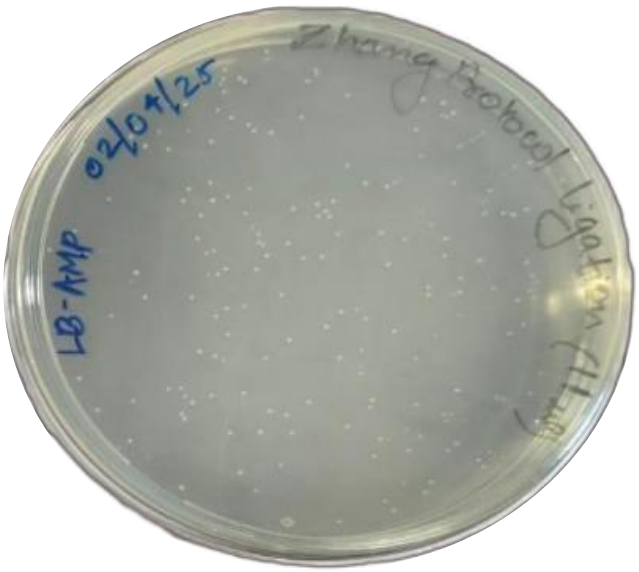


Fig 4.9.1. Positive Test (Recombinant plasmid)

Fig 4.9.2. Negative Control

The presence of many colonies on the plate containing the ligation product demonstrates efficient transformation and suggests that the ligation reaction was successful. The absence of colonies on the negative control plate confirms that contamination was minimal, supporting the specificity of the ligation and transformation process.

The use of ampicillin selection ensured that only *E. coli* cells containing the plasmid (with or without insert) survived, but the negative control result indicates that the insert was necessary for colony formation under these conditions. This further validates the effectiveness of the ligation protocol.

The results confirm successful construction of the recombinant plasmid and efficient transformation into E. coli, providing a solid foundation for downstream applications.

4.10. REPLICA PLATING OF THE RECOMBINANT CLONES

A total of nine distinct E. coli colonies (labelled C1–C9) were observed on the LA plate containing ampicillin. Each colony was streaked individually in a grid pattern, demonstrating isolated and robust growth on the selective medium. The presence of colonies on ampicillin-containing LA plate indicates that the transformed cells successfully acquired the plasmid carrying the ampicillin resistance gene along with the BRCA2E18 AS.

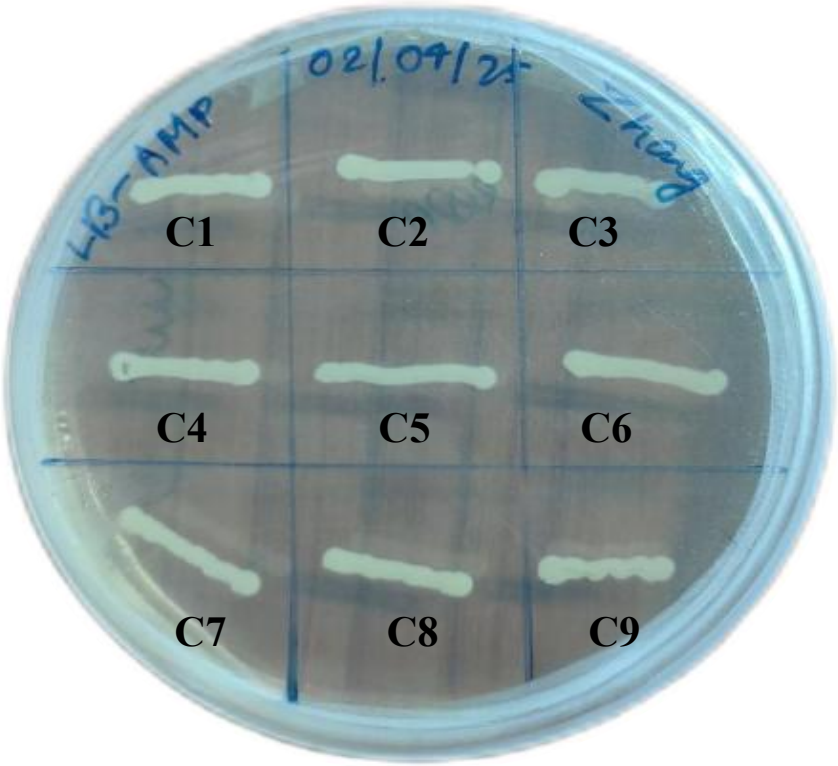


Fig 4.10. BRCA2E18 AS Clones

The appearance of multiple well-separated colonies on the selective LB-ampicillin plate confirms the successful transformation of E. coli with the recombinant plasmid containing the insert i.e. BRCA2E18 AS. The absence of background growth or satellite colonies suggests effective selection and minimal contamination. The clear and distinct streaks for each clone (C1–C9) indicate that the transformation efficiency was sufficient to yield multiple independent clones for downstream analysis.

These results are consistent with a successful ligation and transformation process, as only cells harboring the plasmid with the ampicillin resistance marker are expected to survive and grow on the plate. The availability of several independent clones allows for further screening (such as colony PCR, plasmid extraction, end point PCR and sanger sequencing) to confirm the presence and orientation of the BRCA2E18 AS insert in the plasmid. This step is crucial for ensuring that at least one of the clones carries the correct recombinant construct required for subsequent experiments.

4.11. COLONY PCR OF RECOMBINANT CLONES

Colony PCR was set up as detailed in Table 3.2.13.2 and performed on four selected E. coli colonies (C1–C4) using specific primers. U6 Forward (5’ GCCTATTTCCCATGATTC 3’) as the forward primer and BRCA 2 Bottom oligo (5’ AAACACCAGTTCTGCTCCATGAAGC 3’) as the reverse primer were chosen. The primers used are insert specific, targeting a 252 bp amplicon. The PCR products were analysed by 1.5 % agarose gel electrophoresis alongside a 1 kb DNA ladder and a negative control (no colony template). Distinct bands corresponding to approximately 252 bp were observed for all four colonies (C1, C2, C3, C4), while no band was detected in the negative control lane. This indicates successful amplification of the target insert from each tested colony.

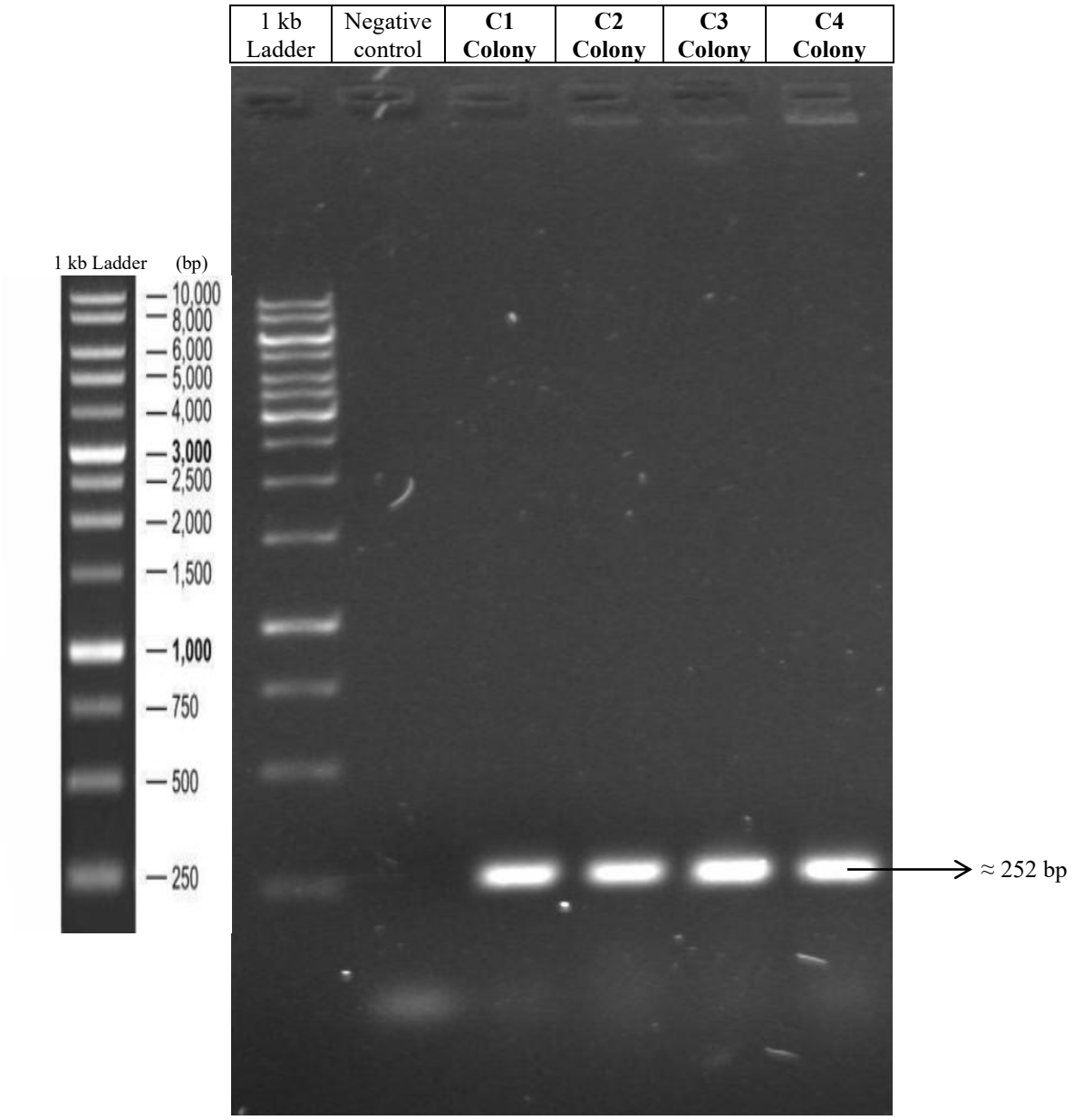


Fig 4.11: Gel electrophoresis of Colony PCR

Lane 1: 1 kb DNA ladder (molecular weight marker)

Lane 2: Negative control (No colony added)

Lane 3: C1 colony (Colony PCR)

Lane 4: C2 colony (Colony PCR)

Lane 5: C3 colony (Colony PCR)

Lane 6: C4 colony (Colony PCR)

The presence of clear, specific bands at the expected size (~252 bp) in all four colony lanes demonstrates that each selected colony has incorporated the recombinant plasmid containing the BRCA2E18 AS insert. The absence of amplification in the negative control confirms the specificity of the PCR and rules out contamination or nonspecific amplification.

These results validate the effectiveness of both the ligation and transformation steps, as multiple independent colonies were shown to contain the desired construct. The successful amplification from all tested colonies suggests a high efficiency of the cloning procedure, enabling confident selection of positive clones for downstream applications such as plasmid isolation and Sanger sequencing.

4.12. PLASMID ISOLATION FROM THE RECOMBINANT CLONES

Plasmid isolation was successfully performed from two E. coli colonies (C1 and C2) containing the recombinant plasmid (insert + vector). The following results were obtained:

1. Colony C1 clone:
    - Concentration: 93.2 ng/µl
    - A260/280 ratio: 1.79
2. Colony C2 clone:
    - Concentration: 106.5 ng/µl
    - A260/280 ratio: 1.82

This result demonstrates efficient isolation of high-quality recombinant plasmid DNA from both C1 and C2 colonies. The measured concentrations (93.2 ng/µl for C1 and 106.5 ng/µl for C2) are suitable for downstream applications such End-point PCR and sanger sequencing. The A260/280 ratios (1.79 and 1.82) confirm that the DNA preparations are relatively pure and free from significant RNA or protein contamination.

The absence of RNA contamination is crucial for accurate downstream molecular biology experiments, as RNA can interfere with enzymatic reactions and quantification. The successful amplification of the insert by PCR further validates that the ligation and transformation steps were effective and that the recombinant construct is present in the isolated plasmids. The isolated plasmids showed no RNA contamination. End-point PCR using a designed primer confirmed the presence of the insert, indicating successful ligation.

Overall, these findings confirm the successful construction and isolation of the recombinant plasmid. Multiple clones with similar results also increase confidence in the reproducibility and reliability of the cloning procedure.

4.13. PRIMER DESIGNING FOR END POINT PCR

The results demonstrate an effective workflow for primer design, combining bioinformatics resources available online like, Addgene (https://www.addgene.org/62988/), NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and SnapGene (https://www.snapgene.com/) to ensure specificity and optimal primer placement. The careful selection of the sequence region 150 bp away from BbsI sites minimizes the risk of amplifying vector backbone or unwanted regions, thereby increasing the specificity of the PCR.

The use of NCBI Primer-BLAST enabled the generation and evaluation of multiple primer pairs based on product size, melting temperature, GC content, and self-complementarity. Primer Pair 3 was selected due to its favourable characteristics and compatibility with the intended amplification region.

Primer pair 3 -

Forward primer: 5'- AATTTCTTGGGTAGTTTGCAGTT -3'
Reverse primer: 5'- CACGCGCTAAAAACGGACTA -3'

Following criteria were considered for designing the primers which will be vector specific that will be used in end point PCR of our recombinant plasmid:

- **Sequence specificity**: The primers were designed to target specific regions of the PX459 (pSpCas9(BB)-2A-Puro) vector, particularly 150bp away from the BbsI restriction sites.

- **Product size**: A defined PCR product size 250-300 bp was specified in the NCBI Primer-BLAST tool.
- **Primer length**: The reverse primer is 20 nucleotides long (5' CACGCGCTAAAACGGACTA 3'), which is within the optimal range for PCR primers.
- **GC content**: For Primer Pair 3, The reverse primer has a GC content of 50.00%, which falls within acceptable parameters.
- **Melting temperature (Tm)**: The primers were designed with compatible Tm values i.e. 58.64°C for reverse primer.
- **Self-complementarity**: Low self-complementarity scores (4.00 for forward primer and 4.00 for reverse primer) to minimize primer-dimer formation.
- **3' self-complementarity**: Values of 0.00 for forward primer and 2.00 for reverse primer, reducing the risk of primer-dimer formation at the critical 3' end.
- **Location relative to functional elements**: The primers were positioned to span relevant regions of the vector, including the U6 promoter and gRNA scaffold regions.

The primer design process followed a systematic approach using bioinformatics tools to ensure optimal PCR amplification. The careful selection of Primer Pair 3 from multiple options generated by NCBI Primer-BLAST demonstrates attention to critical parameters that affect PCR success. The verification of reverse primer location using SnapGene provides additional confidence in the design, ensuring that the primers will amplify the intended region of the PX459 vector.

Only the reverse primer was needed for end point PCR to check the integrity and sequence of the Recombinant plasmid incorporated with our BRCA2E18 AS insert which will be specific to the vector.

4.14. END-POINT PCR

End point PCR was set up as detailed in **Table 3.2.16.2** and performed using the recombinant plasmids isolated from clone C1 and C2 clones as template DNA. It was done using specific primers. U6 Forward (5' GCCTATTTCCCATGATTC 3') as the forward primer and U6 Reverse (5' CACGCGCTAAAAACGGACTA 3') as the reverse primer were chosen. The primers used are vector specific, which are used to check the integrity and sequence of the plasmid. The expected amplicon size was 405 bp. The PCR products, along with a 1 kb DNA ladder and a negative control (no colony), were analysed by 1.5 % agarose gel electrophoresis

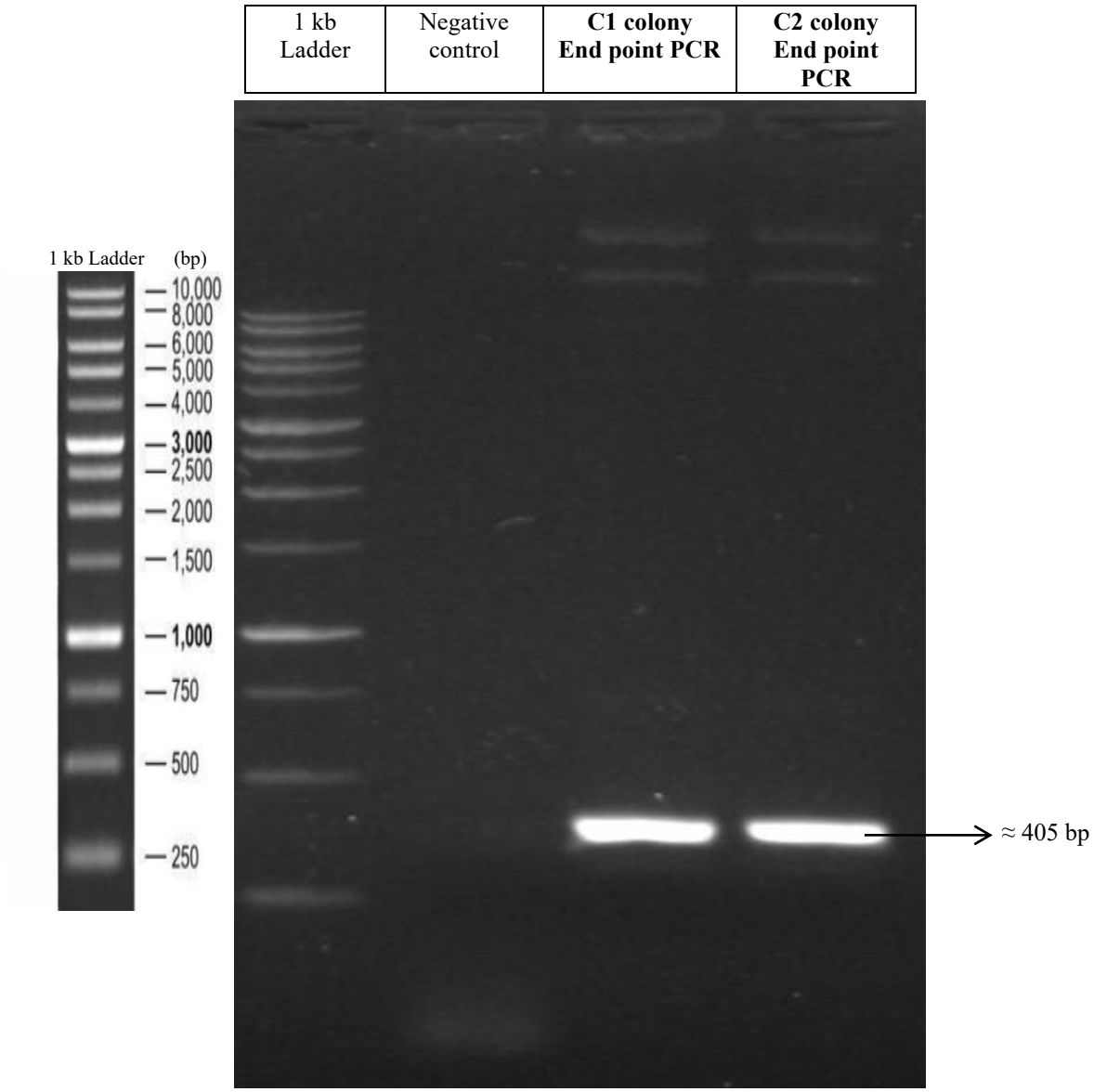


Fig 4.14: Gel electrophoresis of End point PCR

The successful amplification of a 405 bp fragment from both plasmid samples (C1 and C2) confirms the presence of the target insert within the recombinant plasmid. The absence of bands

in the negative control demonstrates the specificity of the PCR reaction and the lack of contamination. These results indicate that the ligation and transformation steps were effective, as both tested colonies contain the correct recombinant construct. The clear and specific bands, without smearing or non-specific amplification, also reflect the quality of the primer design and the efficiency of the PCR conditions. This experiment validates the cloning strategy and confirms that the selected colonies.

4.15. SANGER SEQUENCING OF THE RECOMBINANT PLASMID

Sanger sequencing was performed on two recombinant clones (C1 and C2) to confirm the presence and accuracy of the BRCA2E18 insert. The clones were named **as BRCA2E18 AS** clone 1 **for (**C1 clone) and **BRCA2E18 AS clone 2 for (**C2 clone).

Following are the resulting electropherograms for both the clones after sanger sequencing.

1. DNA Sequence (Electropherogram) of BRCA2E18 AS clone 1

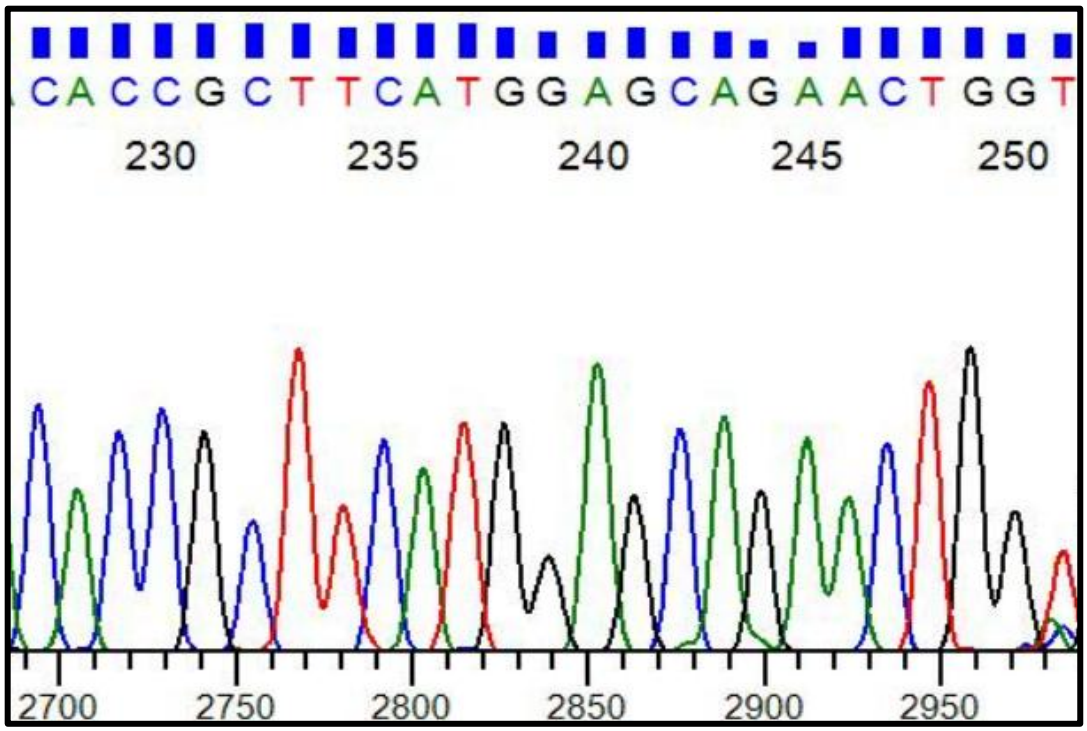


Fig 4.15.1: Electropherogram of BRCA2E18 AS clone 1

The sequence in the electropherogram **Fig.4.16.1** clearly displays the expected BRCA2E18 top oligo sequence i.e. (5' CACCGCTTCATGGAGCAGAACTGGT 3') from position 227 to 251, corresponding to a 25-base pair insert.

2. DNA Sequence (Electropherogram) of BRCA2E18 AS clone 2

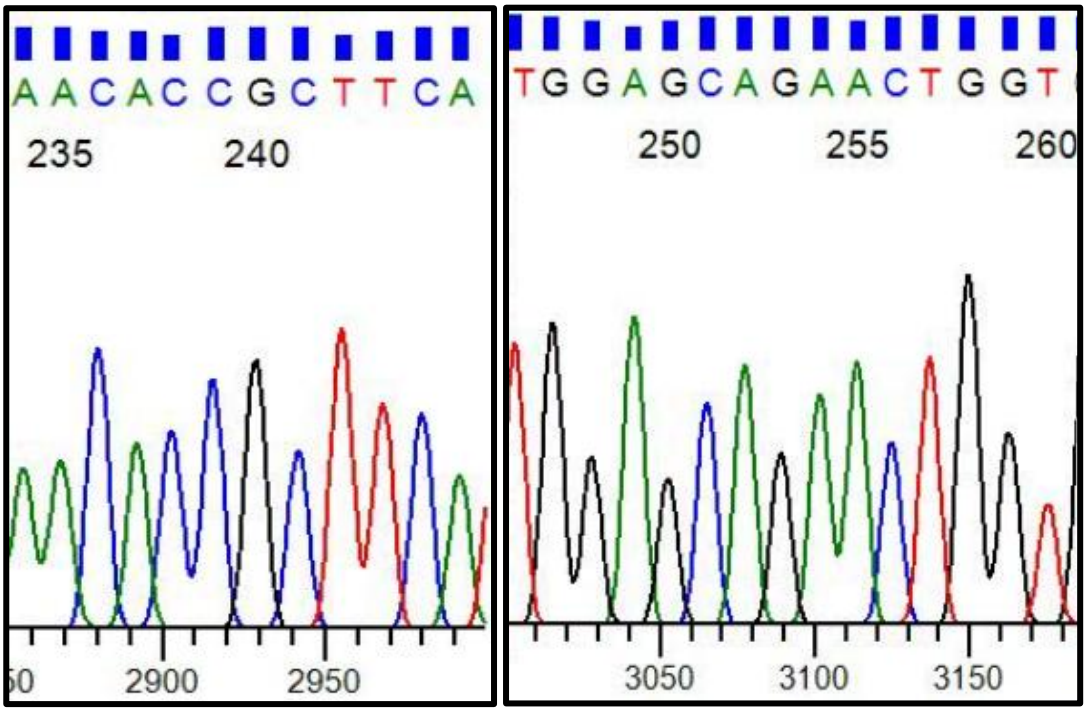


Fig 4.15.2: Electropherogram of BRCA2E18 AS clone 2

The sequence in electropherogram **Fig 4.16.2** is the same, also shows the correct BRCA2E18 top oligo sequence (5' CACCGCTTCATGGAGCAGAACTGGT 3') from position 236 to 260, also confirming a 25-base pair insert.

The Sanger sequencing results confirm the successful insertion and correct sequence of the 25 bp BRCA2E18 AS insert in both recombinant clones (C1 and C2). The sequences obtained from both clones perfectly match the designed insert, with no evidence of mutations or rearrangements. These findings validate the accuracy of the cloning procedure and demonstrate that the desired BRCA2E18 AS sequence was faithfully incorporated into the plasmid in both cases. The high-quality chromatograms further support the integrity of the recombinant constructs. It proves that the desired sgRNA was successfully cloned making these clones suitable for downstream functional studies or further molecular applications

5. CONCLUSION

CONCLUSION

The development of a CRISPR-Cas9-based sgRNA expression system that targets BRCA2 gene is a complex process that combines precise bioinformatics design with cutting-edge molecular biology methods. The Cas9 nuclease and a single guide RNA (sgRNA) are necessary for the CRISPR-Cas9 mechanism, which is used by this system. Through complementary base pairing, the engineered sgRNA guides the Cas9 nuclease to the precise target location on the DNA, in this case, the BRCA2 gene.

For this system to be constructed successfully, careful sgRNA designing is required, ensuring both high on-target efficacy and low off-target effects. Utilising specialised instruments, this entails locating distinct 20-nucleotide sequences next to a Protospacer Adjacent Motif (PAM) sequence usually NGG that is necessary for Cas9 binding and cleavage.

Following sgRNA design, the cloning process involves several key steps, including vector digestion, ligation, transformation, colony selection, and validation. Vectors, such as PX459, are prepared by restriction digestion to create compatible overhangs for the sgRNA insert. The sgRNA insert is then ligated into the digested vector, followed by transformation into competent E. coli cells (DH5α). Colony PCR is used as a quick screening technique to find colonies that have the sgRNA insert in the recombinant plasmid. Sanger sequencing is then used to confirm the sgRNA sequence.

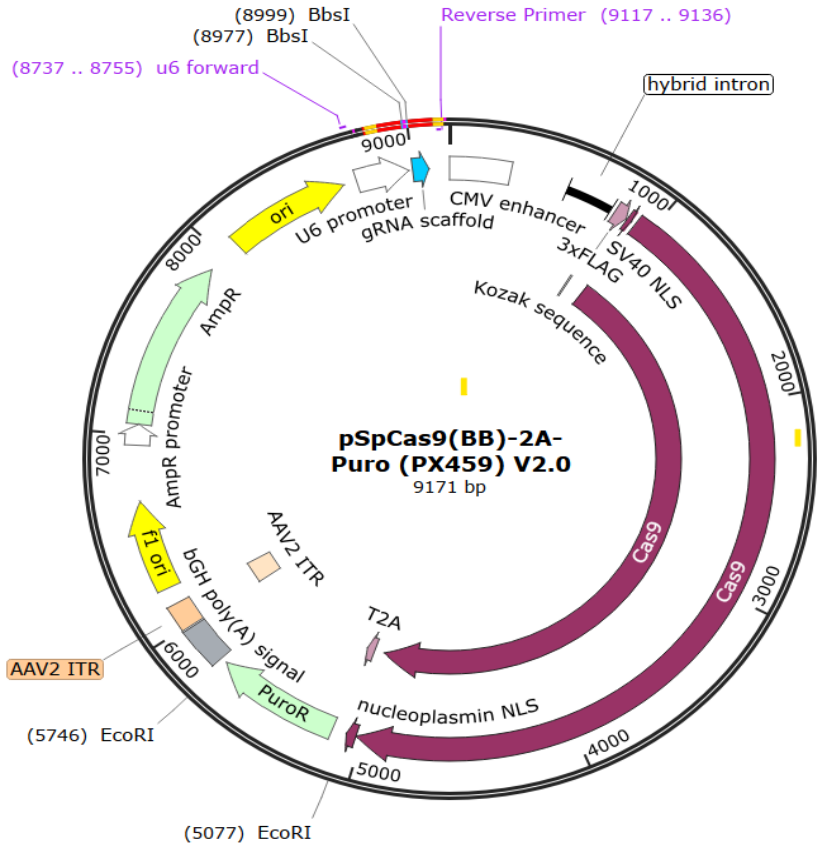
There are many applications for the genome-editing tool CRISPR/Cas-9 in biotechnology, agriculture, and medicine, among other domains. The successful construction of an sgRNA expression system targeting BRCA2 exon 18 provides a powerful tool for targeted gene disruption and functional studies. This system can be used to investigate the effects of BRCA2 mutations on cellular processes, model disease mechanisms, and explore potential therapeutic interventions. By precisely targeting BRCA2 gene, researchers can induce double-stranded breaks (DSBs), which are then repaired by non-homologous end joining (NHEJ), often leading to insertions or deletions (indels) that disrupt the gene's function.

Overall, the meticulous construction and validation of the sgRNA expression system are crucial for reliable and specific genome editing. This technology holds immense potential for advancing our understanding of BRCA2's role in cancer development and for developing novel therapeutic strategies.

6. APPENDIX

APPENDIX

- **Plasmid Map of the Vector (PX459)**



- **Vector specific primer designing region (SnapGene)**

- **BRCA 2 exon 18 FASTA Sequence**

5’ATATGATACGGAAATTGATAGAAGCAGAAGATCGGCTATAAAAAAGATAATGGA
AAGGGATGACACAGCTGCAAAAACACTTGTTCTCTGTGTTTCTGACATAATTTCAT
TGAGCGCAAATATATCTGAAACTTCTAGCAATAAAACTAGTAGTGCAGATACCCAA
AAAGTGGCCATTATTGAACTTACAGATGGGTGGTATGCTGTTAAGGCCCAGTTAGA
TCCTCCCCTCTTAGCTGTCTTAAAGAATGGCAGACTGACAGTTGGTCAGAAGATTA
TTCTTCATGGAGCAGAACTGGTGGGCTCTCCTGATGCCTGTACACCTCTTGAAGCC
CCAGAATCTCTTATGTTAAAG 3’

- **PX459 FASTA Sequence 150 bp away from BbsI SITES**

5’ATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTT
TTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG
ATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGGTCTTCGAGAAGA
CCTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
AAAAAGTGGCACCGAGTCGGTGCTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTA
AAATAAGGCTAGTCCGTTTTTAGCGCGTGCGCCAATTCTGCAGACA 3’

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LINKS (URL's)

a) https://www.ncbi.nlm.nih.gov/gene/675
b) https://chopchop.cbu.uib.no/
c) https://www.addgene.org/62988/
d) Addgene: CRISPR Guide
e) https://www.addgene.org/crispr/zhang/
f) https://www.ncbi.nlm.nih.gov/tools/primer-blast/
g) https://media.addgene.org/cms/filer_public/6d/d8/6dd83407-3b07-47db-8adb-4fada30bde8a/zhang-lab-general-cloning-protocol-target-sequencing_1.pdf
h) https://www.snapgene.com/
i) https://www.synthego.com/guide/how-to-use-crispr/sgrna

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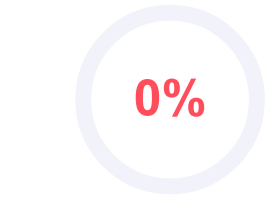

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ABSTRACT

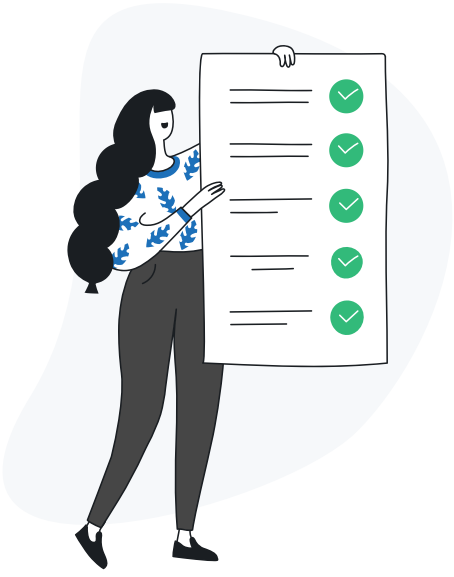
The BRCA2 gene plays a critical role in maintaining genomic stability through homologous recombination-mediated DNA repair. Mutations in the BRCA2 gene are strongly associated with the increased susceptibility to breast, ovarian, and other cancers. Recent advancements in gene-editing technologies, particularly the CRISPR-Cas9 system, offer a promising approach to precisely target and modify disease-related genes. Central to the specificity of CRISPR-Cas9 is the single-guide RNA (sgRNA), which directs Cas9 to the intended genomic locus. This study aimed to construct an efficient sgRNA expression system specifically targeting the BRCA2 gene, to establish a platform for future therapeutic applications or functional studies.

The BRCA2 gene sequence was retrieved from NCBI and analysed to identify suitable protospacer adjacent motif (PAM) sites. Multiple candidate sgRNA sequences were designed using bioinformatics tools such as CHOPCHOP and assessed for off-target potential and on-target efficiency. The most suitable sgRNA was synthesized and cloned into a CRISPR-Cas9 expression plasmid vector, i.e, pSpCas9(BB)-2A-Puro (PX459). The recombinant construct was then transformed into DH5α Escherichia coli, and positive colonies were screened using colony PCR using insert-specific and vector-specific primers. The presence and integrity of the insert was further validated through Sanger sequencing.

The results confirmed successful construction of the sgRNA expression system with high target specificity toward BRCA2. This study demonstrates a systematic approach to designing and constructing a gene-specific CRISPR-Cas9 tool, laying the groundwork for downstream applications.

KEYWORDS –

BRCA2 gene, homologous recombination-mediated DNA repair, CRISPR-Cas9 system, single-guide RNA (sgRNA), Protospacer Adjacent Motif (PAM), expression plasmid vector, DH5α Escherichia coli, colony PCR, Sanger sequencing.



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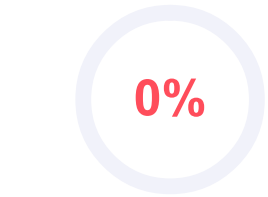

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INTRODUCTION

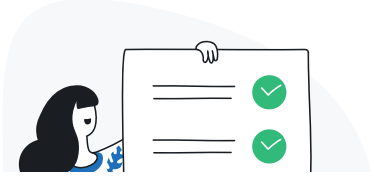
Cancer remains a major global health concern, accounting for millions of deaths annually. Among various genetic contributors to cancer development, the BRCA2 (Breast Cancer Type 2 Susceptibility Protein) gene plays a pivotal role in DNA repair and tumor suppression. BRCA2 is located on chromosome 13q13.1 and encodes a protein that maintains genomic integrity through its involvement in homologous recombination (HR), a key mechanism in the repair of double-stranded DNA breaks (DSB). Loss-of-function mutations in BRCA2 compromise HR, pushing cells toward error-prone repair mechanisms such as non-homologous end joining (NHEJ), thereby increasing the likelihood of chromosomal abnormalities and oncogenic transformation. BRCA2 germline mutations are linked to a higher risk of pancreatic, ovarian, prostate, and breast cancers. The lifetime risk of breast cancer may be 69% higher for women with mutant BRCA2 variants. Consequently, BRCA2 has become an important target for both diagnostic and therapeutic research.

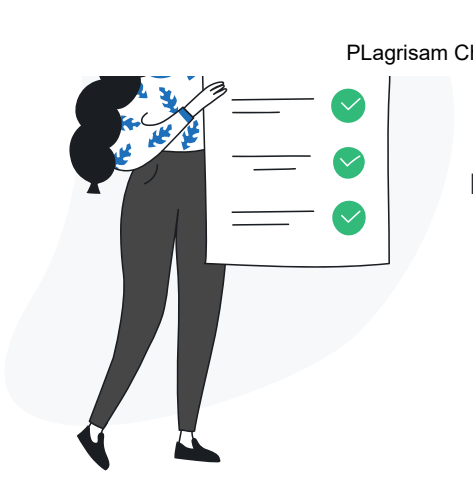
The advent of genome editing technologies has revolutionized the ability to investigate gene function and develop gene-based treatments. Among these, the CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR associated protein 9) system has emerged as a powerful, programmable gene-editing platform. Derived from the adaptive immune system of Streptococcus pyogenes, CRISPR-Cas9 utilizes a single guide RNA (sgRNA) that allows precise targeting and modification of specific DNA sequences in eukaryotic cells. The single-guide RNA (sgRNA), which is at the very core of this system, uses complementary base pairing to guide the Cas9 endonuclease to the target DNA sequence. It comprises of a 20-nucleotide spacer sequence that is complementary to the target DNA and has a scaffold region that binds to Cas9. This sgRNA–Cas9 complex induces double-stranded breaks at the desired site, enabling gene disruption, repair, or replacement.

For CRISPR-mediated gene editing to be successful, an efficient sgRNA must be designed. The specificity and efficiency of the sgRNA directly determine the accuracy of the gene editing process and minimize off-target effects. A properly constructed sgRNA expression system allows for stable and controlled expression of the guide RNA in host cells, thus facilitating targeted genome modifications.

This dissertation focuses on the construction of a sgRNA expression system that targets a specific region in exon 18 of the BRCA2 gene, with a hypothesis of enabling precise gene manipulation for research or therapeutic purposes. The rationale behind this study lies in the potential application of gene-editing systems for knocking out dysfunctional alleles in cancer-prone individuals. Additionally, the system developed here may serve as a foundational tool for further studies into the functional domains of BRCA2 and its interaction with other proteins involved in DNA repair pathways. The goal is to design a high-efficiency, low off-target sgRNA sequence, and incorporate it into a CRISPR-Cas9 expression plasmid i.e. pSpCas9(BB)-2A-Puro (PX459) which is cloned with the help of a competent cell DH5α Escherichia coli, suitable for future gene editing applications. The approach involves bioinformatic analysis to identify potential PAM (Protospacer Adjacent Motif) sites within the BRCA2 coding sequence, selection and validation of optimal sgRNA candidates, and molecular cloning to generate the final expression construct. The project encompasses several key steps: bioinformatic analysis of the BRCA2 gene to identify suitable target sites with adjacent PAM sequences retrieved from NCBI, design and selection of high-specificity sgRNA candidates using established algorithms like CHOPCHOP, cloning of the selected sgRNA sequence into a CRISPR-Cas9 expression vector using SnapGene, Designing of primers using bioinformatic tools like Addgene, NCBI primer BLAST and SnapGene, validation of the construct through molecular techniques including colony PCR, End point PCR and Sanger sequencing.

In conclusion, the targeted manipulation of BRCA2 using CRISPR-Cas9 represents a significant step toward personalized genetic medicine. This research provides a blueprint for constructing customized sgRNA systems for any gene of interest. As the field of genome engineering continues to evolve, the ability to design and validate effective sgRNA constructs will remain a cornerstone of future therapeutic developments.





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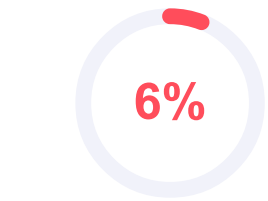

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3.2 METHODS 3.2.1. SINGLE GUIDE RNA (sgRNA) DESIGNING TARGETING BRCA2 GENE. Single guide RNA (sgRNA) design is a critical step in CRISPR-Cas9 genome editing, as it determines the specificity and efficiency of the system. A scaffold sequence that attaches to the Cas9 protein and a 20-nucleotide spacer sequence that directs Cas9 to the target DNA site make up a synthetic RNA molecule known as a sgRNA. Effective sgRNA design involves selecting a target sequence adjacent to a protospacer adjacent motif (PAM), typically "NGG" for SpCas9, and minimizing off-target effects by ensuring sequence uniqueness in the genome. Bioinformatics tools such as CHOPCHOP are commonly used to design sgRNAs with high on-target activity and low off-target potential. Requirements: • Internet access • Target DNA sequence in BRCA 2 gene (in FASTA format) • sgRNA designing tool (CHOPCHOP) Procedure: 1. Retrieval of the Target Gene Sequence: a. Locating BRCA2 on NCBI: • A Google search was conducted to access the NCBI website. • In the NCBI search bar, the term “BRCA2” was entered. b. Choosing the Appropriate Transcript: • From the list of results, the BRCA2 transcript identified by RefSeq ID NM_000059.4 was selected. • The corresponding GenBank entry was reviewed to obtain comprehensive sequence data. c. Extracting the Exon 18 Sequence: • The sequence for Exon 18 was located within the GenBank entry and its FASTA format was copied for downstream analysis. 2. Designing sgRNA Using CHOPCHOP: a. Accessing CHOPCHOP: • Google was used to navigate to the CHOPCHOP web tool. b. Input of Target Sequence: • The FASTA sequence for BRCA2 Exon 18 was pasted into the “Target” field on the CHOPCHOP interface. c. Configuration of Parameters: • The species was set to Homo sapiens. • The application was specified as knock-out. d. Identification of Target Sites: • The "Find Target Sites!" button was clicked to initiate the sgRNA design process. 3. Evaluation and Selection of sgRNA Candidate a. Result Interpretation: • CHOPCHOP generated a list of potential sgRNA target sites within the input sequence, each ranked based on predicted specificity and efficiency. • Information displayed for each sgRNA included: o Target sequence o Genomic coordinates o GC content o Self-complementarity o Predicted activity o Off-target analysis b. Choosing the Optimal sgRNA: • An sgRNA was selected based on: o High predicted cleavage efficiency o Minimal off-target effects o Appropriate GC content (ideally 40–60%) o Low self-complementarity to reduce the likelihood of secondary structure formation 4. Documentation and Synthesis of sgRNA The highest-ranked sgRNA was chosen and converted into two oligonucleotides designed for cloning into the CRISPR-Cas9 vector pSpCas9(BB)-2A-Puro V2.0 (PX459). Overhangs compatible with BbsI restriction sites were added to enable directional cloning. The oligos were named as follows: • BRCA2E18 Top Oligo: 5’-CACCGCTTCATGGAGCAGAACTGGT–3’ [25-nt sgRNA sequence] • BRCA2E18 Bottom Oligo: 5’-AAACACCAGTTCTGCTCCATGAAGC–3’ [25-nt sgRNA sequence] These oligonucleotides were ordered from _____ using the names listed above. 3.2.2. PREPARATION OF COMPETENT CELLS (DH5α) Competent cells are cells which are modified in such a manner that they can easily take up foreign DNA. There are several different methods to make cells competent like electroporation and treatment with calcium ions. Cells are made competent through calcium chloride method by suspending them in a solution containing concentration of calcium and thus creating pores in bacterial cells to take up the foreign DNA. Competency is better suited for cells when they are in their log phase. Requirements: • E. coli strain [DH5α] • Luria-Bertani (LB) medium (Table 3.1.4) • Centrifuge [] • Incubator [] • Solutions for competent cell preparation- COMPONENTS AMOUNT Transformation Buffer [] Table 3.2.2: Components needed for preparation of competent DH5α cell. Procedure: 1. A single colony of E. coli DH5α was picked from the culture plate and inoculated into a 50 ml tube containing 5 ml of LB broth. This culture was incubated overnight at 37°C with shaking at 130 rpm. 2. From the overnight culture, 500 µl was transferred into a fresh 50 ml tube containing 5 ml of LB medium. The culture was incubated at 37°C on a shaker set to 250 rpm until it reached an optical density (OD_{600}) between 0.4 and 0.6. 3. A total of 1.5 ml from the secondary culture was aliquoted into two separate 1.5 ml microcentrifuge tubes. 4. The cells were centrifuged at 10,000 rpm for 1 minute at room temperature. 5. After centrifugation, the supernatant was completely removed, and the cell pellets were resuspended in 600 µl of pre-chilled, sterile transformation buffer. 6. The resuspended cells were kept on ice for 10–15 minutes. 7. Following the cold incubation, the cells were centrifuged again at 10,000 rpm for 1 minute at room temperature. 8. The supernatant was discarded, and the cell pellets were resuspended in 200 µl of pre-chilled sterile transformation buffer, making them ready for transformation. 3.2.3. TRANSFORMATION OF PLASMID (PX459) INTO COMPETENT DH5α E. COLI CELLS Transformation is a method through which bacterium incorporate genes from their surroundings. Some bacteria’s have natural ability to take up foreign DNA. The bacteria are made competent to take up the foreign DNA in laboratories. DH5α E. coli cells can incorporate plasmids from their surroundings through transformation. The competent cells are given heat shock at 42 °C for about 90 seconds. Requirements: • Plasmid PX459 (Table 3.1.1.) • E. coli competent cells [DH5α] (Table 3.1.2.) •

Appropriate Antibiotic (Ampicillin) (Table 3.1.3.) • Luria-Bertani medium (LB) (Table 3.1.4.) • Luria-Bertani agar (LA) (Table 3.1.5) • Incubator • LAF Procedure: 1. 2µl of plasmid PX459 was added into 200 ul of DH5α competent cells prepared previously 3.2.2 in a microcentrifuge tube in LAF, then incubated in ice for 10 min. 2. Then the tubes were placed in a water bath that had been preheated to 42ºC for 90 seconds (heat shock). 3. The tubes were rapidly transferred on ice, and the cells were allowed to chill for 5 min. 4. Then 800 µl of LB medium was added into the tube and incubated for 90 min in an incubator at 37ºC on a shaker at >150 rpm. 5.

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This culture was incubated overnight at 37°C with shaking at 130 rpm. 2.
https://www.fda.gov/media/181588/download

Requirements: • Plasmid PX459 (Table 3.1.1.) • E.
https://www.addgene.org/62988

The tubes were rapidly transferred on ice, and the cells were allowed to chill for 5 min. 4.
https://www.nature.com/articles/s41586-024-07041-8

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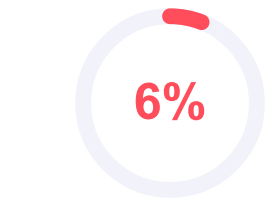

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1. Transformed colonies (PX459 culture plate) was observed the next day and further procedures were carried out. 3.2.4. SINGLE COLONY STREAK PLATE The single colony streak plate method is a microbiological technique used to isolate pure colonies of microorganisms from a mixed culture. In this project, a small amount (single colony) of PX459 bacterial sample was streaked from the culture plate across the surface of an LA plate containing ampicillin in a specific pattern 1. Transformed colonies from a single colony were observed the next day. 3.2.5. BACTERIAL PLASMID ISOLATION (PX459) Isolation of plasmid is carried out by alkaline lysis to break open the cell. The bacterial suspension is first treated with a resuspension buffer to degrade the cell wall. Then, the lysed cell suspension is subjected to an alkaline lysis buffer comprising of Sodium Dodecyl Sulphate (SDS) (anionic surfactant) and a strong base like sodium hydroxide (NaOH). The anionic detergent cleaves the phospholipids of the membrane and denatures the proteins which play an important role in maintain the structure of the cell membrane. The NaOH's alkaline condition leads to denaturation of the plasmid and genomic DNA. Then a neutralization solution is added which contains the acidic potassium acetate that decreases the alkaline condition of the mixture and hence under the acidic condition the hydrogen bonds between complementary bases is restored. The small plasmid DNA anneals quickly owing to its circular and covalent nature whereas the huge genomic DNA cannot properly anneal. Thus, the plasmid remains in the suspension and the denatured genomic DNA and other proteins along with the SDS associate with each other by virtue of hydrophobic interactions and can be separated out by centrifugation as a pellet. The cleared lysate is then subjected to 100% ethanol to retrieve a concentrated plasmid solution. Requirements: • Overnight grown bacterial cells incorporated with PX459 taken from single colony streak plate. • Plasmid DNA purification kit, [SLS RESEARCH PRIVATE LIMITED] • Incubator • Centrifuge • LAF • Nuclease free water Procedure: 1. The bacterial cells incorporated with our plasmid (PX459) were cultured overnight in an incubator at 37ºC in a 50 ml tube containing 5ml LB medium with ampicillin on a shaker at 125rpm. 2. Next day, in LAF the cell suspension was transferred into a 1.5 ml microcentrifuge tube which was centrifuged at 10,000 rpm for 3 min in RT and the supernatant was discarded. 3. To the pelleted cells 250 µl of P1 buffer provided in kit was added and pellet was completely re-suspended by gentle mixing. 4. Then 250 µl of P2 buffer provided in kit was added and thorough mixing was carried out by inverting the tubes 6-10 times 5. Then, 350 µl of PN3 buffer provided in kit was added and mixing was carried out by inverting the tubes 6-10 times. 6. The mixture was then centrifuged at 10,000 rpm for 10 min at RT. 7. The SLS plasmid column was placed with a 2 ml collection tube and the supernatant was decanted into the column. 8. The column was centrifuged at 10,000 rpm for 1 min at RT and flow through was discarded. 9. This was followed by addition of 500 µl of PW1 buffer provided in kit and then the column was centrifuged at 10,000rpm for 1 min at RT. Flow through was discarded 10. The column was washed by adding 700 µl of PW2 buffer (Wash buffer with ethanol) provided in kit and centrifuged at 10,000 rpm for 1 min at RT. Flow through was discarded and column was placed in centrifuge for dry spin at 10,000 rpm for 30 seconds at RT. 11. The SLS RESEARCH PRIVATE LIMITED plasmid column was placed onto a new 1.5 ml microcentrifuge tube. 12. To elute the plasmid DNA a total of 50 µl of nuclease free water was added into the column where firstly 30 µl of NFW was added onto the column and incubated for 5 mins and centrifuged at 10,000 rpm for 1 min and then 20 µl of NFW was added onto the column, incubated for 5 min and then centrifuged at 10,000 rpm for 1 min at RT. 13. The small amount of eluted plasmid DNA was analysed on 1.5 % agarose gel in TAE buffer along with a 1 kb DNA marker. 14. The concentration of the isolated PX459 plasmid was measured using a Nanodrop 3.2.6. AGAROSE GEL ELECTROPHORESIS . In this procedure an electrical field is applied to move the negatively charged DNA, towards the positively charged electrode, through the agarose gel matrix. The smaller DNA fragments move faster than the larger fragments owing to their size. A DNA ladder, having different DNA fragments of known length, is also run alongside the gel to compare the size of the DNA sequence of interest. Ethidium bromide, an intercalating agent (intercalates into the major groove of DNA), is added to the agarose solution before the gel is cast. It fluoresces under Ultraviolet (UV) light and thus the gel can be viewed using an UV transilluminator or photographed in Gel-DOC apparatus. Requirements: • DNA samples • Agarose powder [Sisco Research Laboratories Pvt. Ltd. (SRL)] • Ethidium Bromide [] • DNA molecular weight marker (1 kb ladder) • 10X TAE buffer [] Procedure: 1. The gel apparatus was cleaned using distilled water and wiped dry using clean tissue paper. 2. The gel tray was properly sealed on both the open ends with cello tape to avoid leakage and comb was inserted in place. 3. To prepare 1.5% gel, 30ml of TAE buffer was taken in a flask and 0.45 gm of agarose was added to it. 4. The mixture was then subjected to boiling in a microwave till the agarose completely dissolved in the buffer. 5. After cooling the mixture sufficiently, 2 µl of ethidium bromide (Et-Br) solution was added and mixed gently, to avoid any bubble formation. 6. The liquid mixture was then carefully poured into the gel tray and allowed to solidify. 7. After

20 min, the gel tray was placed

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Then, 350 µl of PN3 buffer provided in kit was added and mixing was carried out by inverting the tubes 6-10 times. 6.
https://era.ed.ac.uk/bitstream/handle/1842/8063/Rodriguez2013.pdf%3Fsequence%3D2%26isAllowed%3Dy

then centrifuged at 10,000 rpm for 1 min at RT. 13.
https://pmc.ncbi.nlm.nih.gov/articles/PMC3792701

A DNA ladder, having different DNA fragments of known length, is also run alongside the gel to compare the size of the DNA sequence of interest.
https://bio.libretexts.org/Bookshelves/Microbiology/Microbiology_(OpenStax)/12%253A_Modern_Applications_of_Microbial_Genetics/12.02%253A_Visualizing_and_Characterizing_DNA

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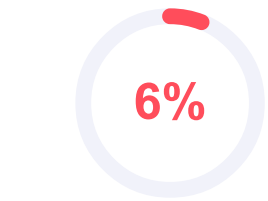

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3.2.7. EcoRI RESTRICTION DIGESTION OF PX459 PLASMID (Confirmatory test)
Restriction digestion is the specific cleavage of the DNA using enzymes called as restriction enzymes such as EcoRI. EcoRI restriction enzymes cut a double stranded DNA, at two places, within a specific site referred to as the restriction recognition sequences. This test was performed as a confirmatory test to check the presence of our desired plasmid PX459 in E. coli
DH5α cells.
Requirements:
• Vector (plasmid PX459)
• 10X buffer. [Thermo Scientific]
• Nuclease free water.
• Water bath
• EcoRI restriction enzyme [Thermo Scientific]
ENZYME RECOGNITION SEQUENCE CUT SITES
EcoRI (5unit/µl) 5'…GAATTC…-3'
5'…CTTAAG…3' i.5077
ii.5764
Table 3.2.7.1: List of restriction enzyme used.
Procedure:
1.
COMPONENTS VOLUME CONCENTRATON
Plasmid PX459 (205.8 ng/µl) 2 µl 411.6 ng
EcoRI enzyme (5unit/µl) 0.5 µl 2.5 unit
10x Buffer 1.5 µl
NFW 16 µl
Total Reaction volume 20 µl
Table 3.2.7.2: Reaction set up for restriction digestion of PX459 with EcoRI.
2. The mixture was incubated in water bath at 37ºC for 2 hours.
3. A small amount of digested DNA was resolved on 1.5% agarose gel with 1X TAE buffer.
3.2.8 ANNEALING AND PHOSPHORYLATION OF OLIGOMERS
To prepare double-stranded DNA inserts for cloning, single stranded oligomers annealing and phosphorylation are essential processes. To enable hybridisation and the formation of double-stranded DNA, complementary single-stranded oligonucleotides are heated and then gradually cooled during the annealing step. After annealing, T4 polynucleotide kinase (PNK) is used to phosphorylate the oligos, adding phosphate groups to their 5' ends to facilitate effective ligation into plasmid vectors. The oligos are correctly paired and chemically ready for insertion into a digested plasmid PX459 backbone.
Requirements:
• BRCA2E18 top oligomer (100 µM)
• BRCA2E18 bottom oligomer (100 µM)
• 10x PCR (Taq) buffer
• T4 polynucleotide kinase (PNK)
• 10x PNK buffer
• PCR machine
• Water bath
Procedure:
3.2.8.1. Annealing of oligomers

3.2.8.1. Annealing of oligomers

1.

COMPONENTS VOLUME AND CONCENTRATION

BRCA2E18 Top oligomer (100 µM)

(5’ CACCGCTTCATGGAGCAGAACTGGT 3’) 2.5 µl (250µM)

BRCA2E18 Bottom oligomer (100 µM)

(5’ AAACACCAGTTCTGCTCCATGAAGC 3’) 2.5 µl (250µM)

10x PCR (Taq) buffer 5 µl

NFW 40 µl

Total Reaction volume 50 µl

Table 3.2.8.1a: Reaction set up for annealing through gradient temperature.

2. The reaction mixture was then subjected to annealing through gradient temperature as mentioned below using a PCR machine:

Step No. Temperature (ºC) Time (minutes)

1 95 2

2 90 5

3 85 5

4 80 5

5 75 5

6 70 5

7 65 5

8 60 5

9 55 5

10 50 5

11 45 5

12 40 5

13 35 5

14 25 5

15 4 ∞

Table 3.2.8.1b: Conditions for annealing through gradient temperature.

3.2.8.2. Phosphorylation of oligomers

1.

COMPONENTS VOLUME AND CONCENTRATION

Annealed Oligomer (5 µM) 44 µl (220 µM)

T4 polynucleotide kinase (PNK) 1 µl

10X PNK buffer 5 µl

Total Reaction volume 50 µl

Table 3.2.8.2: Reaction set up for phosphorylation of oligomers.

2. The reaction mixture was incubated at 37ºC for 2 hours in a water bath.

3. BRCA2E18 oligomers both top and bottom were annealed and phosphorylated. Annealed oligomers are ready to be used as the insert for ligation into the plasmid PX459 and was named as BRCA2E18 AS.

3.2.9. BBSI RESTRICTION DIGESTION OF PX459 PLASMID The specific cleavage of DNA by restriction enzymes, such as BbsI, is known as restriction digestion. Within a particular site known as the restriction recognition sequences, restriction enzymes cut double-stranded DNA twice. Palindromes typically make up such sequences. While some restriction enzymes produce blunt ends, others make incisions that result in staggered ends, which aid in future effective ligation. BbsI is a hexacutter type IIs restriction enzyme which produces sticky ends that aid in the cloning of desired DNA inserts, such as the sticky-ended BRCA2E18 AS.

Requirements:

• Vector (plasmid PX459)

• 10X buffer. [Thermo Scientific]

• Nuclease free water.

• Water bath

• BbsI restriction enzyme [Thermo Scientific]

ENZYME RECOGNITION SEQUENCE CUT SITES

BbsI (10unit/µl) 5'… GAAGACNN…3'

5’…NNCAGAAG…3’ i.8977

ii.8999

Table 3.2.9.1: List of restriction enzyme used.

Procedure:

1.

COMPONENTS VOLUME CONCENTRATION

Plasmid PX459 (205.8 ng/µl) 9.8 µl 2016.84 ng

BbsI enzyme 1 µl 10 unit

10x BbsI buffer 5 µl

NFW 34.2 µl

Total Reaction volume 50 µl

Table 3.2.9.2: Reaction set up for restriction digestion of PX459 with BbsI

2. The mixture was incubated in water bath at 37ºC for 2 hours.

3. A small amount of digested DNA (PX459) was resolved on 1.5% agarose gel with 1X TAE buffer.

4. Rest of the digested plasmid was then purified using PCR purification kit (SLS), where the digested plasmid was treated with PW1 buffer in a spin column and centrifuged at 10,000rpm for 1 min at RT.

5. Flow through was discarded, and PW2 buffer was added into spin column and centrifuged at 10,000rpm for 1 min at RT.

6. Flow through was discarded, after an empty spin the digested plasmid was eluted in 50 µl of NFW.

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2. The reaction mixture was incubated at 37ºC for 2 hours in a water bath.
https://www.rsc.org/suppdata/c7/tb/c7tb00212b/c7tb00212b1.pdf

Table 3.2.8.2: Reaction set up for phosphorylation of oligomers.
https://www.youtube.com/watch%3Fv%3Dq5X7N2o7jBo%26pp%3D0gcJCdgAo7VqN5tD

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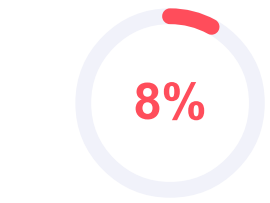

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Total Words: 1000 **Total Characters:** 6008 **Plagiarized Sentences:** 4.16 **Unique Sentences:** 47.84 (92%)

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3.2.10. LIGATION OF INSERT BRCA2E18 AS WITH BbsI DIGESTED VECTOR PX459 Ligation is catalysed by the enzyme known as DNA ligase. It catalyses the formation of a phosphodiester bond between 3'-hydroxyl and 5'-phosphate groups of the DNA backbone. DNA ligation is a normal process in cell and is a part of repair machinery to repair nicks in single or both the strands of DNA. In this study, ligation procedure is used to join the BRCA2E18 AS and BbsI digested plasmid PX459 Requirements: • Vector (BbsI digested and purified plasmid PX459) • Insert (BRCA2E18 AS) • T4 DNA ligase • Ligation Buffer • Nuclease free water • LAF Procedure: 1. The following ligation mixture was set up in LAF for ligation in a 100 µl microcentrifuge tube. COMPONENTS VOLUME CONCENTRATION Vector DNA (16 ng/µl) 4 µl 64 ng Insert DNA (4.4µM) 1 µl 4.4 µM T4 DNA Ligase 1 µl Ligation Buffer 1 µl NFW 4 µl Total Reaction volume 11 µl 2. The ligation mixture was incubated at RT for 4 hours. 3. DH5α (E. coli) competent cells were prepared same as 3.2.2. 3.2.11. TRANSFORMATION OF RECOMBINANT PLASMID INTO THE COMPETENT (DH5α) Requirements: • Recombinant Plasmid (Insert + Vector) • E. coli competent cells [DH5α] (Table 3.1.2.) • Appropriate Antibiotic (Ampicillin) (Table 3.1.3.) • Luria-Bertani medium (LB) (Table 3.1.4.) • Luria-Bertani agar (LA) (Table 3.1.5) • Incubator • LAF Procedure: 1. In LAF 11µl of ligation mixture (recombinant plasmid) was added into 200ul of E. coli competent cells in a 500 µl microcentrifuge tube, then incubated in ice for 10 min. 2. Then the tubes were placed in a water bath that had been preheated to 42ºC for 90 seconds (heat shock). 3. The tubes were rapidly transferred on ice and allowed the cells to chill for 5 min. 4. 800 µl of LB medium was added into the tube and incubated in an incubator for 90 min at 37ºC on a shaker at >150 rpm. 5. The cells were then centrifuged at 10,000 rpm for 1 min in RT. 6. Approximately 700 µl of supernatant was discarded, remaining were re-suspended with pellet. 7. The re-suspended cell suspension was spread plated on LA plate containing an appropriate antibiotic i.e. Ampicillin in LAF. 8. The plates were then incubated overnight in an incubator at 37ºC. 9. Transformed colonies containing our recombinant plasmid was observed next day and further procedures were carried out. 3.2.12. REPLICA PLATING OF TRANSFORMED COLONIES method for moving bacterial colonies from one agar plate to another in the same spatial arrangement is called "replica plating of transformed colonies." It is frequently used to screen for phenotypes, like antibiotic resistance. By contrasting growth patterns on selective media, replica plating enables the identification of clones that have successfully incorporated the recombinant plasmid in the case of transformed colonies the recombinant plasmid master plate are streaked onto fresh plates using a sterile inoculation loop, guaranteeing uniform colony positioning for simple comparison. Requirements: • Colonies of transformed bacterial cells • Luria-Bertani Agar (LA) Plate containing ampicillin. • Sterile inoculating Loop. • Incubator • LAF Procedure: 1. Using a marker, nine quadrants were marked starting from C1 – C9 on the back side of the Luria-Bertani agar plate. 2. In LAF, a total of nine colonies from the transformed colonies incorporated with the recombinant plasmid master plate were selected and streaked onto each quadrant respectively. 3. Selected transformed colonies containing our recombinant plasmid was observed next day and further procedures were carried out. 3.2.13. COLONY PCR technique for detecting whether insert DNA (BRCA2E18 AS) is present in the plasmid (PX459) is colony PCR. Colony PCR is used to ascertain whether the insert is orientated correctly. A small portion of a colony is picked and directly used as the template in a PCR reaction without prior DNA purification. The cells are lysed by heat or through the PCR process itself, releasing DNA for amplification. Specific primers are used to amplify target regions, confirming the presence or orientation of the insert. This technique is commonly used after transformation to quickly identify positive clones. Using gel electrophoresis comparison of the bands produced is done with a DNA marker, the presence or absence of the PCR amplicons is ascertained. Requirements: • Template - Colonies from replica plate. (C1, C2, C3 and C4) • 10x PCR buffer • DNTP • Taq DNA polymerase • NFW • Thermal Cycler • PCR Cabinet • Primers (Insert Specific) PRIMERS SEQUENCE CONCENTRATION U6 Forward 5’ GCCTATTTCCCATGATTCC 3 10 µM BRCA2E18 bottom oligomer 5’ AAACACCAGTTCTGCTCCATGAAGC 3’ 10 µM Procedure: 1. Inside the PCR cabinet he following components were added in a 1.5 ml microcentrifuge tube. COMPONENTS 1 REACTION (20µl) 6 REACTIONS (Master Mix) NFW 14.75 µl 88.5 µl 10x PCR buffer 2 µl 12 µl DNTP 2 µl 12 µl U6 Forward Primer 0.5 µl 3 µl BRCA2E18 Bottom Oligo 0.5 µl 3 µl Taq 0.25 µl 1.5 µl 2. 20 µl of master mix was taken in 5 PCR tubes each. 3. Using different tips, C1, C2, C3 and C4 colonies were mixed into four different PCR tubes respectively. 4. Last PCR tube was kept as a control where no colonies were mixed. 5. STEP No. STEP TEMPRETURE TIME I Initial denaturation 94ºC 5 min II Denaturation 94 ºC 30 sec III Annealing 60 ºC 30 sec IV Extension 72 ºC 48 sec V Final extension 72 ºC 5 min VI Hold 25 ºC 1 min Step II – IV were repeated for 30 cycles 6. PCR products were resolved on 1.5% agarose gel in 1X TAE buffer. 3.2.14. RECOMBINANT PLASMID ISOLATION The recombinant plasmids having the desired insert (BRCA2E18 AS) can be extracted from transformed bacterial cells using a technique called bacterial

plasmid isolation 3.2.5. Bacteria containing the plasmid are usually cultured in LB medium, and then the cells are broken open by alkaline lysis, which denatures the proteins and chromosomal DNA. After that, the plasmid DNA is purified by spin-column techniques. For subsequent uses like End Point PCR (primers specific

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Colony PCR is used to ascertain whether the insert is orientated correctly.
https://www.neb.com/en-us/applications/cloning-and-synthetic-biology/dna-analysis/colony-pcr

Requirements: • Template - Colonies from replica plate.
https://www.scribd.com/document/409150505/Replica-plating

COLONY PCR technique for detecting whether insert DNA (BRCA2E18 AS) is present in the plasmid (PX459) is colony PCR.
https://www.neb.com/en-us/applications/cloning-and-synthetic-biology/dna-analysis/colony-pcr%3Fsrsltid%3DAfmBOop8c3P1xvDytOEaHmiaI9_BWiavW3lZoLm6rXrsczYExJK0Y0Lc

STEP TEMPRETURE TIME I Initial denaturation 94ºC 5 min II Denaturation 94 ºC 30 sec III Annealing 60 ºC 30 sec IV Extension 72 ºC 48 sec V Final
https://www.chegg.com/homework-help/questions-and-answers/time-cycles-step-temp-94-c-5-min-initial-denaturation-denaturation-94-c-30-sec-45-sec-30-3-q61188523

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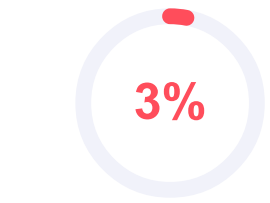

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3.2.14. RECOMBINANT PLASMID ISOLATION

The recombinant plasmids having the desired insert (BRCA2E18 AS) can be extracted from transformed bacterial cells using a technique called bacterial plasmid isolation 3.2.5. Bacteria containing the plasmid are usually cultured in LB medium, and then the cells are broken open by alkaline lysis, which denatures the proteins and chromosomal DNA. After that, the plasmid DNA is purified by spin-column techniques. For subsequent uses like End Point PCR (primers specific to the vector) to confirm the presence of recombinant plasmid and sanger sequencing, this isolated recombinant plasmid can be employed.

Requirements:

• Overnight grown bacterial cells incorporated with recombinant plasmid. (2 colonies chosen from the colonies used in colony PCR)

• Incubator

• Plasmid DNA purification kit, [SLS RESEARCH PRIVATE LIMITED]

• Centrifuge

• Nanodrop

• Nuclease free water

Procedure:

1. The bacterial cells from 2 colonies C1 and C2 were grown overnight in an incubator at 37ºC in 5ml LB medium with ampicillin.

2. Next day, the cell suspension of the colonies was centrifuged in 2 different microcentrifuge tubes (1.5 ml) respectively at 10,000 rpm for 3 min in RT and the supernatant was discarded.

3. To the pelleted cells 250 µl of P1 buffer provided in kit was added and pellet was completely re-suspended by gentle mixing.

4. Then 250 µl of P2 buffer was added provided in kit and thorough mixing was carried out by inverting the tubes 6-10 times.

5. Then, 350 µl of PN3 buffer was added provided in kit and mixing was carried out by inverting the tubes 6-10 times.

6. centrifuged at 10,000 rpm for 10 min at RT.

7. The SLS plasmid column was placed with a 2 ml collection tube and the supernatant was decanted into the column

8. column was centrifuged at 10,000 rpm for 1 min at RT and flow through was discarded.

9. This was followed by addition of 500 µl of PW1 buffer provided in kit and then the column was centrifuged at 10,000rpm for 1 min at RT. Flow through was discarded

The column was washed by adding 700 µl of PW2 buffer

centrifuged at 10,000 rpm for 1 min

10. column was placed onto a new 1.5 ml microcentrifuge tube.

11. To elute the recombinant plasmid DNA for both the colonies a total of 50 µl of nuclease free water was added into the column where firstly 30 µl of NFW was added onto the column and incubated for 5 mins and centrifuged at 10,000 rpm for 1 min and then 20 µl of NFW was added onto the column, incubated for 5 min and then centrifuged at 10,000 rpm for 1 min at RT.

12. The eluted recombinant plasmid DNA of colony C1 and C2 concentration was measured using a nanodrop.

13. After then, end-point PCR conducted to confirm successful ligation using a designed primers specific to the vectors.

3.2.15. PRIMER DESIGNING

In the end-point PCR (Polymerase Chain Reaction) process, primer design is an essential step that entails producing brief single-stranded DNA sequences complementary to the target DNA

region.

attach to the template DNA and give DNA polymerase a place to start synthesising new DNA. They are typically 18–25 nucleotides long. Effective primer design ensures specificity, optimal melting temperature (Tm), balanced GC content (40–60%), and minimal secondary structures like hairpins or dimers. Primers are frequently designed using software programs like NCBI Primer-BLAST to ensure precise and effective amplification. In this project the primers that are designed are spec

Requirements:

• Internet access

• Internet access

Target DNA sequence of
(PX459) V2.0

• Primer design software (NCBI Primer-BLAST)

• Oligo analysis tool (SnapGene)

Procedure:

1. Retrieval of PX459 plasmid sequence.

The full plasmid sequence was retrieved in FASTA (. fasta) format from the Addgene website: https://www.addgene.org/62988/. The SnapGene file downloaded and saved it locally.

2. The sequenced was opened in SnapGene and the target region i.e. 150bp away from the BbsI sites was identified and copied for designing primers.

3. The copied target DNA sequence was pasted in the NCBI Primer-BLAST: https://www.ncbi.nlm.nih.gov/tools/primer-blast/ tool template box.

4. The parameters for designing the primers were adjusted.

5. “Get Primers” was clicked to generate a list of suggested primer pairs.

6. The primer pairs were reviewed, and primer pair 3 was chosen.

7. Only the reverse primer was copied and pasted in SnapGene plasmid PX459 file, the binding of primer to the plasmid PX459 was visualised and the amplicon size was determined which will be produced after end point PCR of the isolated recombinant plasmid.

8. The reverse primer was named as U6 reverse primer and was ordered.

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6. centrifuged at 10,000 rpm for 10 min at RT.

https://www.med.upenn.edu/robertsonlab/assets/user-content/documents/miniprep-for-plasmid-extraction.pdf

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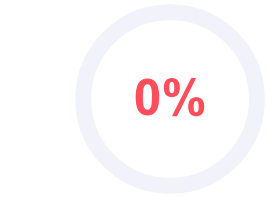

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3.2.16. END POINT PCR (POLYMERASE CHAIN REACTION)
End-point PCR is a method for amplifying the target DNA sequences and can be frequently used to verify the integrity and presence of recombinant plasmid. Primers in this situation are made to target areas that surround the inserted gene or a junction where the vector and insert meet. 1.5% Agarose gel electrophoresis is used to analyse the PCR product following amplification. The successful cloning and insertion of the desired DNA fragment into the plasmid is indicated by the presence of a band of the expected size, which validates the plasmid's recombinant nature.
Requirements:
• Template – Plasmid DNA of the colonies C1 and C2.
• 10x PCR buffer
• DNTP
• Taq DNA polymerase
• NFW
• Thermal Cycler
• PCR Cabinet
• Primers (Vector Specific)
PRIMERS SEQUENCE CONCENTRATION
U6 Forward 5’ GCCTATTTCCCATGATTCC 3 10 µM
U6 Reverse 5’ CACGCGCTAAAAACGGACTA 3’ 10 µM
Procedure:
1.
COMPONENTS 1 REACTION (20µl) 4 REACTIONS (Master Mix)
NFW 14.75 µl 59 µl
10x PCR buffer 2 µl 8 µl
DNTP 2 µl 8 µl
U6 Forward Primer 0.5 µl 2 µl
U6 Reverse Primer 0.5 µl 2 µl
Taq 0.25 µl 1 µl
2. 20 µl of master mix was taken in 3 PCR tubes each.
3. 2 µl of template was added into 2 different PCR tubes.
4. Last PCR tube was kept as a control where no colonies were mixed.
5. The following PCR conditions were utilized for PCR amplification:

STEP No. STEP TEMPRETURE TIME
I Initial denaturation 95 ºC 5 min
II Denaturation 95 ºC 30 sec
III Annealing 56 ºC 30 sec
IV Extension 72 ºC 48 sec
V Final extension 72 ºC 5 min
VI Hold 25 ºC 1 min
Step II – IV were repeated for 30 cycles
6. PCR products were resolved on 1.5% agarose gel in 1X TAE buffer.

3.2.17. SANGER SEQUENCING

Sanger sequencing is a key technique used in the thesis project to verify the accuracy of recombinant plasmid constructs. After cloning and transformation, selected plasmid DNA samples are sequenced using sanger sequencing to confirm the correct insertion and orientation of the target gene. This method provides high-quality, base-level confirmation, ensuring that no mutations or errors occurred during amplification or ligation steps. The sequencing results are aligned with the reference sequence to validate the integrity of the recombinant plasmid, supporting the reliability of downstream experiments.

Requirements:

• Plasmid DNA - High-quality, contamination-free DNA extracted from transformed colonies C1 and C2.

• Sequencing Primer – (U6 Forward primer 10μM)

Procedure:

1. 20 μl of plasmid DNA (2μg) was aliquoted into a 1.5 ml microcentrifuge tube for both recombinant plasmids.
2. 10 μl of U6 forward primer (10μM) was aliquoted into a 1.5 ml microcentrifuge tube.
3. Both aliquoted primer and plasmid DNA was sent for sanger sequencing
4. The electropherogram of both plasmids were analysed carefully.

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RESULTS AND DISCUSSION

4.1. sgRNA DESIGNING

The single guide RNA (sgRNA) was designed to target Exon 18 of the BRCA2 gene, a region of interest due to its role in DNA repair and its implication in cancer when mutated. The reference sequence for BRCA2 was retrieved from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/gene/675) to ensure accuracy and relevance to the human genome.

Candidates of sgRNA sequences targeting the BRCA 2 gene were generated using the CHOPCHOP (https://chopchop.cbu.uib.no/)) online tool, which provides potential CRISPR/Cas9 target sites based on user-defined parameters such as target gene, exon region, and PAM sequence specificity.

From the list of sgRNA candidates, the top-ranking sgRNA sequence was chosen as the target sequence i.e. “5’-CTTCATGGAGCAGAACTGGTGGG -3’”. It was selected based on several factors including high on-target score, low predicted off-target effects, and its location within the coding region of exon 18.

The following parameters were used to select the most optimal sgRNA:

• GC content between 40–60% for stable binding and transcription. GC content of our target sequence is 50 %.
• High on-target efficacy score (>50%). The target sequence has a 64.78 % efficiency.
• Minimal predicted off-target effects. The chosen target sequence has 0 off-target.
• Minimal Self- complementarity. The chosen target sequence has 0 self-complementarity
• Proximity to functional exons or critical protein domains of BRCA2

The top-ranking sgRNA sequence was selected and converted into a pair of oligonucleotides for cloning into the CRISPR-Cas9 vector (pSpCas9(BB)-2A-Puro (PX459)). Overhangs compatible with BbsI restriction enzyme sites were added.

• BRCA2E18 Top Oligomer: 5'- CACCGCTTCATGGAGCAGAACTGGT -3’ [25-nt sgRNA sequence]
• BRCA2E18 Bot Oligomer: 5'-AAACACCAGTTCTGCTCCATGAAGC -3' [25-nt sgRNA sequence]

Into the top strand (5′-CACCG...-3′) overhang was added and to the bottom strand (5′-AAAC...-3′) overhang was added.

The inclusion of specific 5 bp overhangs CACCG on the top strand and 4 bp AAAC on the bottom strand facilitates directional cloning and ensures proper orientation within the plasmid.

4.2. PREPARATION OF COMPETENT CELLS (DH5α)

The calcium chloride technique, which changes the porousness of the membrane, to make cells permeable to foreign DNA, was successfully used to create competent E. coli DH5α cells. The cells were prepared as mentioned in the procedure 3.2.2 and cultured until they reached the mid-log phase (OD_{600} ≈ 0.4–0.6), which is when transformation is most likely to occur. To foster competence, the cell pellets were centrifuged, then gently resuspended in a 200 µl of transformation buffer. The cells appeared creamy and uniform, indicating good viability and proper handling throughout the protocol.

The competent cells were stored in 4ºC to avoid any loss of competency. To evaluate competency, a transformation efficiency test was performed using the PX459 plasmid. The resulting number of colonies on LA-ampicillin plates suggested a transformation efficiency within the expected range for chemically competent DH5α cells.

4.3 TRANSFORMATION OF PLASMID PX459

The transformation of E. coli DH5α competent cells with the plasmid PX459 (Cas9 expression vector) was successfully performed using the heat-shock method as mentioned in the procedure of 3.2.3 i.e. 45ºC for 90 sec. Two LB agar plates containing ampicillin (100 µg/mL) were used: one with the transformed cells (experimental plate) and one with untransformed competent cells (negative control).

On the experimental plate (left), numerous well-isolated colonies were observed, indicating successful uptake of the pX459 plasmid, which confers ampicillin resistance. The high colony count suggests that the competent DH5α cells had good transformation efficiency, and the plasmid was successfully maintained and expressed.
In contrast, the negative control plate (right), which contained competent DH5α cells without plasmid DNA, showed no colony growth, confirming that the ampicillin resistance observed on the experimental plate was due to successful transformation with PX459. This also rules out contamination or inherent resistance of the competent cells.
The results validate both the efficiency of the competent cell preparation and the successful incorporation of the PX459 plasmid, which can now be used as aan expression vector for downstream applications such as cloning of our BRCA2E18 insert for CRISPR/Cas9-mediated genome editing.

4.4. STREAK PLATING OF SINGLE COLONY
A single colony from the transformation plate containing the plasmid DNA (PX459) was streaked on an LB agar plate supplemented with ampicillin to isolate a pure bacterial colony. The purpose of this streak plate was to obtain well-isolated colonies derived from a single transformed cell for downstream applications such as plasmid extraction.

Fig 4.4: Single colony streak plate (PX459)
As shown in the plate image, distinct and well-separated colonies appeared along the streak lines, particularly in the final streak region. This indicates successful isolation of single colonies and confirms that the transformed E. coli cells (DH5α) retained the plasmid containing the ampicillin resistance gene. The growth pattern also suggests good viability and proper handling during streaking.
No contamination or unexpected growth was observed, supporting the purity of the colony used. The presence of discrete colonies derived from a single clone ensures genetic consistency in subsequent experiments. Overall, the streak plate technique effectively yielded isolated colonies, providing a reliable source of monoclonal culture for further procedures to be carried out i.e. making of an expression vector which will be ligated with the BRCA2E18 insert.

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4.5 BACTERIAL PLASMID ISOLATION (PX459)
The Plasmid DNA purification kit [SLS RESEARCH PRIVATE LIMITED], which was previously mentioned in the procedure of 3.2.5, was used to successfully isolate Plasmid DNA PX459 from E. coli DH5α cells using a standard alkaline lysis method. The quality and presence of the plasmid DNA were assessed by resolving it in 1.5% agarose gel electrophoresis.

1 kb Ladder Plasmid DNA

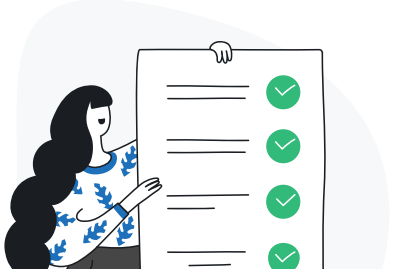
)

Lane 1: 1 kb ladder
Lane 2: PX459 plasmid DNA
The most prominent band corresponds to approximately 9.2 kb, which matches the expected size of the PX459 plasmid.
the 1 kb DNA ladder run alongside provides clear molecular weight reference markers, confirming the approximate size of the plasmid
. Additional bands observed above the main band likely represent different conformations of the plasmid DNA, such as nicked or linearized forms, which is typical for plasmid preparations.
The concentration of the isolated plasmid PX459 was measured using a Nanodrop which turned out to be 205.8 ng/µl. The A260/280 ratio was 1.81. The presence of sharp and distinct bands without smearing indicates that the plasmid DNA was successfully isolated with minimal degradation and without any RNA contamination. This result confirms the successful transformation and replication of the PX459 plasmid in E. coli DH5α cells
4.6. RESTRICTION DIGESTION OF PLASMID PX459 USING EcoRI RESTRICTION ENDONUCLEASE (Confirmatory Test)
To confirm the identity and integrity of the isolated PX459 plasmid from DH5α cells, a restriction digestion was performed using EcoRI restriction endonuclease as detailed in the reaction setup Table 3.2.7.2.
restriction enzyme that recognizes the 5'…GAATTC…3' sequence. EcoRI sequences usually comprise of palindromes
create incisions in such a way that it produces staggered ends. The total restriction recognition sites present in the PX459 is two and are located at 5077 and 5764 position. EcoRI is a hexacutter restriction endonuclease which yields sticky ends and cuts at 2 different sites when incubated at 37ºC. The digested plasmid was analysed by 1.5 % agarose gel electrophoresis alongside uncut plasmid and a 1 kb DNA ladder for size reference.

1 kb Ladder Plasmid DNA EcoRI Digested

1 kb Ladder Plasmid DNA EcoRI Digested

Lane 1: 1 kb
ladder
Lane 2: Uncut PX459 plasmid DNA
Lane 3: Plasmid PX459 DNA digested with EcoRI Restriction endonuclease
The uncut plasmid lane displayed multiple bands corresponding to different plasmid conformations nicked and linear forms, which is typical for undigested plasmid DNA. This pattern is consistent, as previously observed (~ 9.2 kb).
In contrast, the EcoRI digested plasmid showed 2 distinct band near the expected size of 8.4kb and 688 bp, consistent with complete linearization of the plasmid by EcoRI. The disappearance of the multiple conformational bands seen in the uncut sample and the appearance of two linear band confirms that:
• The plasmid was successfully digested, verifying the integrity and identity of the plasmid PX459.
• The DNA is of good quality and is free from inhibitors that could interfere with enzymatic activity.
This digestion serves as a confirmatory test, ensuring that the plasmid isolated is indeed PX459 and that it retains the correct sequence and structure necessary for downstream applications regarding the project.
4.7. ANNEALING AND PHOSPHORYLATION OF BRCA2E18 OLIGOMERS
To generate a double-stranded guide RNA (sgRNA) insert targeting exon 18 of the BRCA2 gene, complementary top and bottom strand oligomers were designed and synthesized. The BRCA2E18 Top Oligomer (5′CACCGCTTCATGGAGCAGAACTGGT-3′) and the BRCA2E18 Bottom oligomer (5′- AAACACCAGTTCTGCTCCATGAAGC -3′) were chosen based on the target sequence identified through CHOPCHOP, which ensures high specificity and minimal off-target activity.
The single stranded oligomers were subjected to a two-step process to finally construct our double stranded BRCA2E18 insert:
1. Annealing
2. Phosphorylation
Annealing of the oligomers was accomplished after the set up detailed in Table 3.1.8.1a by binding the two complementary single-stranded DNA oligomers (BRCA2E18 Top oligomer 100 µM and BRCA2E18 Bottom oligomer 100µM) together to form double-stranded DNA through hydrogen bonding between complementary base pairs. Successful annealing was indicated by the formation of double-stranded oligonucleotides with compatible overhangs for BbsI cloning sites present in the PX459 vector.
phosphorylation of the duplex is done using T4 Polynucleotide Kinase (PNK) to add 5′ phosphate groups
which are necessary for subsequent ligation into the PX459 vector. The final concentration after phosphorylation was 4.4 µM in 50 µl.
The following is the double stranded sgRNA sequence after annealing and phosphorylation which will be our desired insert which was named as BRCA2E18 AS:
5’ CACCGCTTCATGGAGCAGAACTGGT 3’ CGAAGTACCTCGTCTTGACCACAAA 5’
This step is critical for the CRISPR/Cas9 gene editing workflow, as it ensures the designed sgRNA can be properly transcribed and guide the Cas9 endonuclease to the genomic target. The resulting duplexed, phosphorylated oligos were used for ligation into the linearized PX459 vector for transformation into
DH5α.



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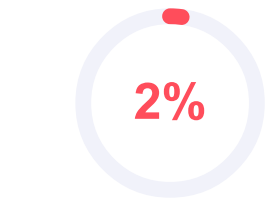

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RESTRICTION ENDONUCLEASE

The goal of digesting the PX459 plasmid with BbsI as detailed in the reaction setup Table 3.2.9.2. was to create compatible sticky ends for cloning the BRCA2E18 AS insert into the plasmid backbone using Target Sequence Cloning Protocol, ZhangLab. BbsI is a Type IIS restriction enzyme that cuts DNA outside of its recognition sequence i.e. 5'…GAAGACNN…3' sequence. BbsI cut site sequences usually comprise of palindromes. The PX459 plasmid was successfully digested with the BbsI restriction enzyme. BbsI is a hexacutter restriction endonuclease which yields sticky ends and cuts at 2 different sites when incubated at 37ºC. BbsI restriction enzymes create incisions at the restriction recognition sites present in the PX459 located at 8977 and 8999 position in such a way that it produces staggered ends onto which our BRCA2E18 AS insert will be ligated. The digested plasmid was analysed by 1.5 % agarose gel electrophoresis alongside uncut plasmid and a 1 kb DNA ladder for size reference.

1 kb Ladder Plasmid DNA BbsI Digested

Lane 1: 1 kb
ladder
Lane 2: Uncut PX459 plasmid DNA
Lane 3: Plasmid PX459 DNA digested with BbsI Restriction endonuclease

This agarose gel image demonstrates the successful restriction digestion of the PX459 plasmid by the BbsI enzyme. The uncut plasmid lane shows a prominent band corresponding to the supercoiled form, along with faint bands representing the possibly nicked forms, which is typical of uncut plasmids.

In contrast, the BbsI-digested lane shows single clear distant band near the expected size 9.1 kb when compared with the 1 kb DNA ladder, indicating linearization or complete digestion of the plasmid. The clarity and sharpness of the band in the digested lane suggest efficient enzyme activity and minimal star activity or degradation. Concentration of digested plasmid was calculated to be 40.33 ng/µl (2016.84ng ÷ 50). The linearized plasmid runs faster compared to the nicked form, confirming that BbsI enzyme successfully cleaved the specific recognition sites in the PX459 vector. PX459 contains BbsI sites flanking the gRNA cloning site, and digestion exposes overhangs compatible with the phosphorylated and annealed BRCA2E18 oligonucleotides. This digestion step is critical in preparing the plasmid for the insertion of target-specific oligonucleotides using Target Sequence Cloning Protocol, ZhangLab.

BbsI digested PX459 vector with our double stranded BARC2E18 AS from 4.7, the digested plasmid was purified to remove any sort of contamination and to isolate pure BbsI digested plasmid for ligation. PCR purification kit (SLS) was used to purify the digested plasmid

contamination and to isolate pure BbsI digested plasmid for ligation. PCR purification kit (SLS) was used to purify the digested plasmid
Before the purification the concentration of the BbsI digested PX459 plasmid was 40 ng/µl (Total = 1600ng in 40µL), but after purification the expected concentration of BbsI digested PX459 plasmid was calculated to be
16 ng/µl
After purification it is considered that half of the DNA will be lost during the process. Before purification 1600 ng of digested DNA was present in 40 µl reaction, which got reduced to 800 ng after purification when eluted in 50 µl NFW thus giving us the concentration of the purified BbsI digested plasmid as 16 ng/µl.
The insert (BRCA2E18 AS) was successfully ligated into the PX459 plasmid vector using a T4 ligase-mediated reaction, as detailed in the reaction setup Table 3.2.10.1
transformed into competent DH5α E. coli cells. Following transformation and plating on LB agar containing ampicillin, numerous colonies were observed on the experimental plate, while no colonies appeared on the negative control plate (vector without insert), as shown in the images.
The presence of colonies on selective media indicates successful uptake of recombinant plasmids by the host cells. Further confirmation of positive clones was performed using colony PCR.
The presence of many colonies on the plate containing the ligation product demonstrates efficient transformation and suggests that the ligation reaction was successful. The absence of colonies on the negative control plate confirms that contamination was minimal, supporting the specificity of the ligation and transformation process.
The use of ampicillin selection ensured that only E. coli cells containing the plasmid (with or without insert) survived, but the negative control result indicates that the insert was necessary for colony formation under these conditions. This further validates the effectiveness of the ligation protocol.
The results confirm successful construction of the recombinant plasmid and efficient transformation into E. coli, providing a solid foundation for downstream applications.
4.10. REPLICA PLATING OF THE RECOMBINANT CLONES
A total of nine distinct E. coli colonies
observed on the LA plate containing ampicillin. Each colony was streaked individually in a grid pattern, demonstrating isolated and robust growth on the selective medium. The presence of colonies on ampicillin-containing LA plate indicates that the transformed cells successfully acquired the plasmid carrying the ampicillin resistance gene along with the BRCA2E18 AS.
multiple well-separated colonies on the selective LB-ampicillin plate confirms the successful transformation of E. coli with the recombinant plasmid containing the insert i.e. BRCA2E18 AS. The absence of background growth or satellite colonies suggests effective selection and minimal contamination. The clear and distinct streaks for each clone (C1–C9) indicate that the transformation efficiency was sufficient to yield multiple independent clones for downstream analysis.
These results are consistent with a successful ligation and transformation process, as only cells harboring the plasmid with the ampicillin resistance marker are expected to survive and grow on the plate. The availability of several independent clones allows for further screening (such as colony PCR, plasmid extraction, end point PCR and sanger sequencing) to confirm the presence and orientation of the BRCA2E18 AS insert in the plasmid. This step is crucial for ensuring that at least one of the clones carries the correct recombinant construct required for subsequent experiments.

Plagiarized Sources

1 kb Ladder Plasmid DNA BbsI Digested
https://www.neb.com/en-us/products/n3232-1-kb-dna-ladder%3Fsrsltid%3DAfmBOorTCvKizzUc6Fd6fFf5E-wDQJ1Ot0xJ2K3Hpq0atD_jX3qj0LfM

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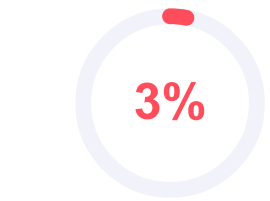

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4.11. COLONY PCR OF RECOMBINANT CLONES
Colony PCR was set up as detailed in Table 3.2.13.2 and performed on four selected E. coli colonies (C1–C4) using specific primers. U6 Forward (5’ GCCTATTTCCCATGATTC 3’) as the forward primer and BRCA 2 Bottom oligo (5’ AAACACCAGTTCTGCTCCATGAAGC 3’) as the reverse primer were chosen. The primers used are insert specific, targeting a 252 bp amplicon. The PCR products were analysed by 1.5 % agarose gel electrophoresis alongside a 1 kb DNA ladder and a negative control (no colony template). Distinct bands corresponding to approximately 252 bp were observed for all four colonies (C1, C2, C3, C4), while no band was detected in the negative control lane. This indicates successful amplification of the target insert from each tested colony.
1 kb Ladder Negative
control C1
Colony C2
Colony C3
Colony C4
Colony

Lane 1: 1 kb DNA ladder (molecular weight marker)
Lane 2: Negative control (No colony added)
Lane 3: C1 colony (Colony PCR)
Lane 4: C2 colony (Colony PCR)
Lane 5: C3 colony (Colony PCR)
Lane 6: C4 colony (Colony PCR)
The presence of clear, specific bands at the expected size (~252 bp) in all four colony lanes demonstrates that each selected colony has incorporated the recombinant plasmid containing the BRCA2E18 AS insert. The absence of amplification in the negative control confirms the specificity of the PCR and rules out contamination or nonspecific amplification.
These results validate the effectiveness of both the ligation and transformation steps, as multiple independent colonies were shown to contain the desired construct. The successful amplification from all tested colonies suggests a high efficiency of the cloning procedure, enabling

the desired construct. The successful amplification from all tested colonies suggests a high efficiency of the cloning procedure, enabling confident selection of positive clones for downstream applications such as plasmid isolation and Sanger sequencing.

4.12. PLASMID ISOLATION FROM THE RECOMBINANT CLONES

Plasmid isolation was successfully performed from two E. coli colonies (C1 and C2) containing the recombinant plasmid (insert + vector). The following results were obtained:

1. Colony C1 clone:
• Concentration: 93.2 ng/µl
• A260/280 ratio: 1.79
2. Colony C2 clone:
• Concentration: 106.5 ng/µl
• A260/280 ratio: 1.82

This result demonstrates efficient isolation of high-quality recombinant plasmid DNA from both C1 and C2 colonies. The measured concentrations (93.2 ng/µl for C1 and 106.5 ng/µl for C2) are suitable for downstream applications such End-point PCR and sanger sequencing. The A260/280 ratios (1.79 and 1.82) confirm that the DNA preparations are relatively pure and free from significant RNA or protein contamination.

The absence of RNA contamination is crucial for accurate downstream molecular biology experiments, as RNA can interfere with enzymatic reactions and quantification. The successful amplification of the insert by PCR further validates that the ligation and transformation steps were effective and that the recombinant construct is present in the isolated plasmids. The isolated plasmids showed no RNA contamination. End-point PCR using a designed primer confirmed the presence of the insert, indicating successful ligation.

Overall, these findings confirm the successful construction and isolation of the recombinant plasmid. Multiple clones with similar results also increase confidence in the reproducibility and reliability of the cloning procedure.

4.13. PRIMER DESIGNING FOR END POINT PCR

The results demonstrate an effective workflow for primer design, combining bioinformatics resources Addgene (https://www.addgene.org/62988/), NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and SnapGene (https://www.snapgene.com/) to ensure specificity and optimal primer placement. The careful selection of the sequence region 150 bp away from BbsI sites minimizes the risk of amplifying vector backbone or unwanted regions, thereby increasing the specificity of the PCR.

The use of NCBI Primer-BLAST enabled the generation and evaluation of multiple primer pairs based on product size, melting temperature, GC content, and self-complementarity. Primer Pair 3 was selected due to its favourable characteristics and compatibility with the intended amplification region.

Primer pair 3 -
Forward primer: 5'- AATTTCTTGGGTAGTTTGCAGTT -3'
Reverse reverse: 5'- CACGCGCTAAAAACGGACTA -3'

Following criteria were considered for designing the primers which will be vector specific that will be used in end point PCR of our recombinant plasmid:

• Sequence specificity: The primers were designed to target specific regions of the PX459 (pSpCas9(BB)-2A-Puro) vector, particularly 150bp away from the BbsI restriction sites.
• Product size: A defined PCR product size 250-300 bp was specified in the NCBI Primer-BLAST tool.
• Primer length: The reverse primer is 20 nucleotides long (5' CACGCGCTAAAACGGACTA 3'), which is within the optimal range for PCR primers.
• GC content: For Primer Pair 3, The reverse primer has a GC content of 50.00%, which falls within acceptable parameters.
• Melting temperature (Tm): The primers were designed with compatible Tm values i.e. 58.64°C for reverse primer.
• Self-complementarity: Low self-complementarity scores (4.00 for forward primer and 4.00 for reverse primer) to minimize primer-dimer formation.
• 3' self-complementarity: Values of 0.00 for forward primer and 2.00 for reverse primer, reducing the risk of primer-dimer formation at the critical 3' end.
• Location relative to functional elements: The primers were positioned to span relevant regions of the vector, including the U6 promoter and gRNA scaffold regions.

The primer design process followed a systematic approach using bioinformatics tools to ensure optimal PCR amplification. The careful selection of Primer Pair 3 from multiple options generated by NCBI Primer-BLAST demonstrates attention to critical parameters that affect PCR success. The verification of reverse primer location using SnapGene provides additional confidence in the design, ensuring that the primers will amplify the intended region of the PX459 vector.

Only the reverse primer was needed for end point PCR to check the integrity and sequence of the Recombinant plasmid incorporated with our BRCA2E18 AS insert which will be specific to the vector.

Plagiarized Sources

Lane 1: 1 kb DNA ladder (molecular weight marker)
https://www.researchgate.net/figure/Agarose-gel-electrophoresis-of-PCR-amplification-of-NP24-gene-The-PCR-product-was_fig1_234094769

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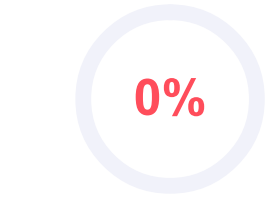

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4.14. END-POINT PCR
End point PCR was set up as detailed in Table 3.2.16.2 and performed using the recombinant plasmids isolated from clone C1 and C2 clones as template DNA. It was done using specific primers. U6 Forward (5’ GCCTATTTCCCATGATTC 3’) as the forward primer and U6 Reverse (5’ CACGCGCTAAAAACGGACTA 3’) as the reverse primer were chosen. The primers used are vector specific, which are used to check the integrity and sequence of the plasmid. The expected amplicon size was 405 bp. The PCR products, along with a 1 kb DNA ladder and a negative control (no colony), were analysed by 1.5 % agarose gel electrophoresis
1 kb Ladder Negative
control C1 colony
End point PCR C2 colony
End point PCR

The successful amplification of a 405 bp fragment from both plasmid samples (C1 and C2) confirms the presence of the target insert within the recombinant plasmid. The absence of bands in the negative control demonstrates the specificity of the PCR reaction and the lack of contamination. These results indicate that the ligation and transformation steps were effective, as both tested colonies contain the correct recombinant construct. The clear and specific bands, without smearing or non-specific amplification, also reflect the quality of the primer design and the efficiency of the PCR conditions. This experiment validates the cloning strategy and confirms that the selected colonies.

4.15. SANGER SEQUENCING OF THE RECOMBINANT PLASMID
Sanger sequencing was performed on two recombinant clones (C1 and C2) to confirm the presence and accuracy of the BRCA2E18 insert. The clones were named as BRCA2E18 AS clone 1 for (C1 clone) and BRCA2E18 AS clone 2 for (C2 clone).
Following are the resulting electropherograms for both the clones after sanger sequencing.
1. DNA Sequence (Electropherogram) of BRCA2E18 AS clone 1

The sequence in the electropherogram Fig 4.16.1 clearly displays the expected BRCA2E18 top oligo sequence i.e. (5’

The sequence in the electropherogram Fig. 4.16.1 clearly displays the expected BRCA2E18 top oligo sequence (5’ CACCGCTTCATGGAGCAGAACTGGT 3’) from position 227 to 251, corresponding to a 25-base pair insert.

2. DNA Sequence (Electropherogram) of BRCA2E18 AS clone 2

The sequence in electropherogram Fig 4.16.2 is the same, also shows the correct BRCA2E18 top oligo sequence (5’ CACCGCTTCATGGAGCAGAACTGGT 3’) from position 236 to 260, also confirming a 25-base pair insert.

The Sanger sequencing results confirm the successful insertion and correct sequence of the 25 bp BRCA2E18 AS insert in both recombinant clones (C1 and C2). The sequences obtained from both clones perfectly match the designed insert, with no evidence of mutations or rearrangements. These findings validate the accuracy of the cloning procedure and demonstrate that the desired BRCA2E18 AS sequence was faithfully incorporated into the plasmid in both cases. The high-quality chromatograms further support the integrity of the recombinant constructs. It proves that the desired sgRNA was successfully cloned making these clones suitable for downstream functional studies or further molecular applications

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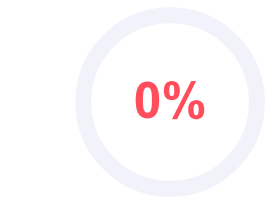

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CONCLUSION

The development of a CRISPR-Cas9-based sgRNA expression system that targets BRCA2 gene is a complex process that combines precise bioinformatics design with cutting-edge molecular biology methods. The Cas9 nuclease and a single guide RNA (sgRNA) are necessary for the CRISPR-Cas9 mechanism, which is used by this system. Through complementary base pairing, the engineered sgRNA guides the Cas9 nuclease to the precise target location on the DNA, in this case, the BRCA2 gene.

For this system to be constructed successfully, careful sgRNA designing is required, ensuring both high on-target efficacy and low off-target effects. Utilising specialised instruments, this entails locating distinct 20-nucleotide sequences next to a Protospacer Adjacent Motif (PAM) sequence usually NGG that is necessary for Cas9 binding and cleavage.

Following sgRNA design, the cloning process involves several key steps, including vector digestion, ligation, transformation, colony selection, and validation. Vectors, such as PX459, are prepared by restriction digestion to create compatible overhangs for the sgRNA insert. The sgRNA insert is then ligated into the digested vector, followed by transformation into competent E. coli cells (DH5α). Colony PCR is used as a quick screening technique to find colonies that have the sgRNA insert in the recombinant plasmid. Sanger sequencing is then used to confirm the sgRNA sequence.

There are many applications for the genome-editing tool CRISPR/Cas-9 in biotechnology, agriculture, and medicine, among other domains. The successful construction of an sgRNA expression system targeting BRCA2 exon 18 provides a powerful tool for targeted gene disruption and functional studies. This system can be used to investigate the effects of BRCA2 mutations on cellular processes, model disease mechanisms, and explore potential therapeutic interventions. By precisely targeting BRCA2 gene, researchers can induce double-stranded breaks (DSBs), which are then repaired by non-homologous end joining (NHEJ), often leading to insertions or deletions (indels) that disrupt the gene's function.

Overall, the meticulous construction and validation of the sgRNA expression system are crucial for reliable and specific genome editing. This technology holds immense potential for advancing our understanding of BRCA2's role in cancer development and for developing novel therapeutic strategies.

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